PB82-224320

Dermatotoxicity: Selected Issues in Testing for Dermal Toxicity, Including Irritation, Sensitization, Phototoxicity, and Systemic Toxicity

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Rockville, MD

Prepared for

Environmental Protection Agency
Washington, DC

Jan 82

U.S. Department of Commerce
National Technical Information Service
NTIS

PB82-224320

United States
Environmental Protection
Agency

Office of Pesticides and
Toxic Substances
Washington, D.C. 20460

EPA-560/11-82-002
January 1982

Pesticides and Toxic Substances

DERMATOTOXICITY

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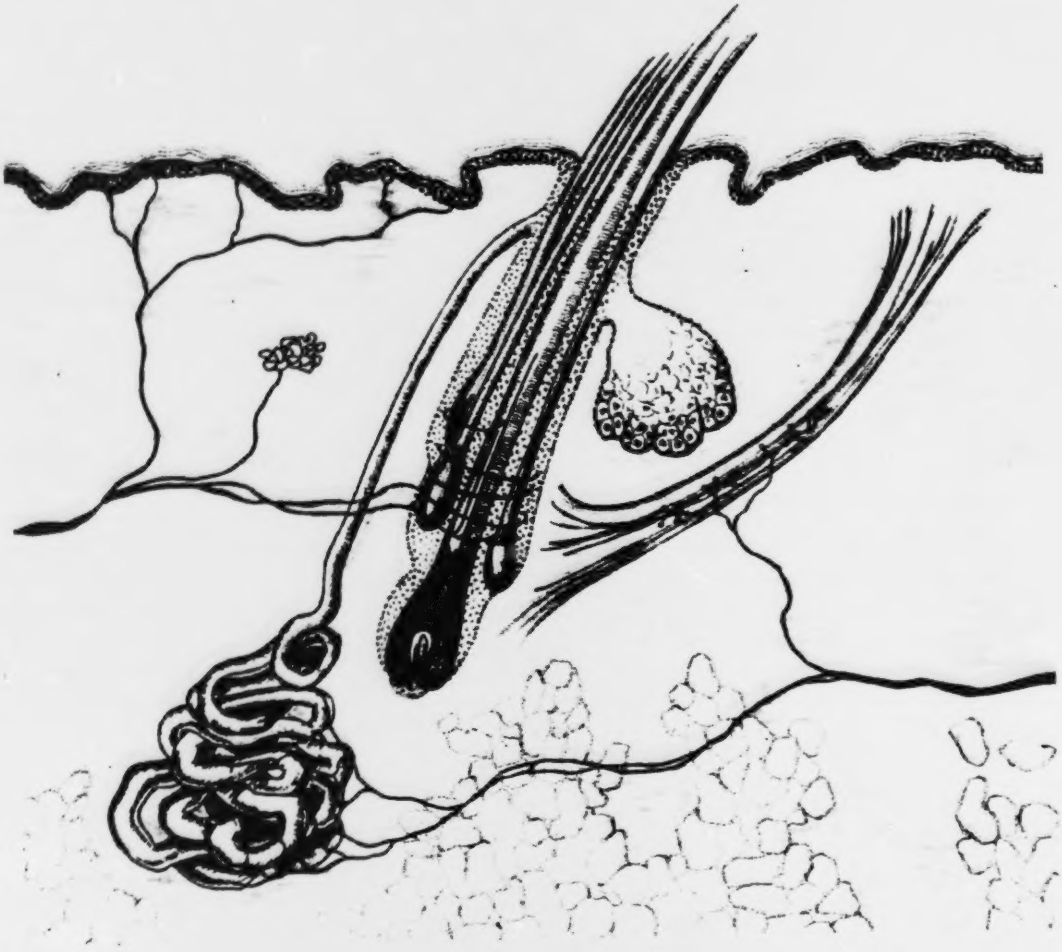

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Preparation of this document was completed prior to the January 22, 1982 effective date of the EPA Administrator's Order 2200 and consequently did not necessarily undergo the peer review procedures described therein. The document received peer review according to procedures in place prior to that date and received complete administrative review.

EPA 560/11-82-002
January 1982

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WASHINGTON D.C. 20460

50272-101

REPORT DOCUMENTATION PAGE

1. REPORT NO.
EPA 560/11-82-002

2.

3. Recipient's Accession No.
PB82-224320

4. Title and Subtitle
DERMATOTOXICITY
Selected Issues in Testing for Dermal Toxicity, Including Irritation, Sensitization, Phototoxicity, and Systemic Toxicity

5. Report Date
January, 1982

6.

7. Author(s)

8. Performing Organization Rept. No.

9. Performing Organization Name and Address
Tracor Jitco, Inc.
1776 East Jefferson Street
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10. Project/Task/Work Unit No.
Work Assignment 02

11. Contract(C) or Grant(G) No.
(C) 68-01-6176
(G)

12. Sponsoring Organization Name and Address
Office of Toxic Substances
U.S. Environmental Protection Agency
Washington, D.C. 20460

13. Type of Report & Period Covered
Final Technical Report

14.

15. Supplementary Notes

16. Abstract (Limit: 200 words)

Four categories of Dermatotoxicity testing are examined: Dermal Irritatio Sensitization, Systemic Toxicity, and Phototoxicity. The rabbit is most widely used for irritation; the guinea pig is also acceptable, as its sensitivity is comparable. Factors affecting dermal irritation include: the degree of occlusion, use of abrasion, the application site, and duration of exposure and observation. This review suggests a tier-like strategy utilizing pH limits and preliminary screening in the hairless mouse may be useful in evaluating irritation potential. Eight guinea pig methods are considered acceptable for determining sensitization; they utilize intradermal and/or epicutaneous routes of administration. The Maximization and Closed Patch tests are most widely used, but no one method has been sufficiently validated to support its selection as the method of choice. Ultraviolet light can alter a non-toxic chemical to one causing direct phototoxicity or allergenicity. Following topical or intraperitoneal application of a compound a test site is irradiated with UV light and reactions are scored. Dermal toxicity testing determines whether substances can be absorbed sufficiently to produce systemic effects. Factors influencing irritation also influence systemic toxicity. Relative rates of percutaneous absorption of a series of compounds can be estimated from LD50 values via different routes. Comparison of dermal LD50's for rabbit and rats shows that more than 75% of the values varied by less than a factor of four. Neither species clearly showed greater sensitivity. Dermal LD50 values were similar for a 24-hour study in rabbits and a 4-hour study in rats.

17. Document Analysis a. Descriptors
Toxic Tolerances; Skin; Absorption; Laboratory Animals; Guinea Pigs; Skin Effect; Toxicology; Photosensitivity; Lethal Dosage; Contact Dermatitis; Allergic Skin Diseases; Skin Test Agents; Tests; Experimental Design; Standards

b. Identifiers/Open-Ended Terms
Dermal Toxicity Testing Methods; Dermatotoxicity; Skin Permeability; Patch Tests; Guidelines; Organization for Economic Cooperation and Development; OECD; Environmental Protection Agency; EPA; Interagency Regulatory Liaison Group; IRLG; Sensitization

c. COSATI Field/Group

18. Availability Statement
Document is available to the public through the National Technical Information Service, Springfield, VA 22151.

19. Security Class (This Report)

20. Security Class (This Page)

21. No. of Pages
155

22. Price

(See ANSI–Z39.18)

See Instructions on Reverse

OPTIONAL FORM 272 (4–77
(Formerly NTIS–35)

TABLE OF CONTENTS

Page

PREFACE vii

EXECUTIVE SUMMARY ix

UNRESOLVED ISSUES AND RESEARCH RECOMMENDATIONS xiii

DERMAL IRRITATION

1.0 SUMMARY 1

2.0 INTRODUCTION 2
2.1 Objective 2
2.2 Definitions 2
2.3 Historical 2
2.4 pH of Test Agents 7

3.0 SPECIES SELECTION 9
3.1 Animal Tests. 9
3.2 Interspecies Comparison 9

4.0 TESTING PROCEDURES 18
4.1 Patch Test Unit 18
4.1.1 Immersion 27
4.1.2 Occlusion 27
4.1.3 Conclusions on Patch Test Unit 28
4.2 Abrasion 29
4.3 Application of Dry Test Substances 33
4.4 Application Site 35
4.5 Exposure and Observation Period 35
4.6 Enhancements to Visual Scoring 37

5.0 REPRODUCIBILITY AND SCORING 38
5.1 Reproducibility 38
5.2 Other Scoring Methods 39
5.3 Recommendations 40

6.0 CONCLUSIONS 41

7.0 REFERENCES 42

TABLE OF CONTENTS (Continued)

Page

TABLES

1-1 Evaluation of Skin Reactions 4
1-2 Primary Irritant Response Categories in the Rabbit . . . 4
1-3 Comparison of Methods Used for Dermal Irritation 5
1-4 Summary of Primary Irritation Scores 10
1-5 Relative Irritancy, Man vs Seven Other Species 13
1-6 Ranking of Skin Irritation Results With Surfactants On Human and Animal Skin 14
1-7 Irritation Response of Human, Guinea Pig, and Rabbit Skin to Chemicals/Household Products 15
1-8 Assessment of Irritation — Mouse Ear 16
1-9 Summary of Testing Procedures Used in Dermal Studies . . 19
1-10 Four-Hour Patch Test Results 30
1-11 Effect of Abrasion on Skin Irritation in Humans 34

FIGURES

1 Patch Test Unit. 25
2 Al Test. 25
3 Finn Chamber 26
4 KI Chamber 26

DERMAL SENSITIZATION

1.0 SUMMARY 47

2.0 INTRODUCTION 49
2.1 Objectives 49
2.2 Scope 49
2.3 Definitions 50
2.4 The Sensitization Test 50
2.5 Methods for Sensitization 50
2.6 Experimental Animal 51

3.0 PREDICTIVE TEST METHODS FOR SKIN SENSITIZATION USING GUINEA PIGS 52
3.1 Draize Test 52
3.2 Freund's Complete Adjuvant Test 56
3.3 Open Epicutaneous Test 57
3.4 Closed Patch Test 58
3.5 Maximization Test 59
3.6 Optimization Test 61
3.7 Split-Adjuvant Test 62
3.8 Footpad Test 64

4.0 COMPARATIVE RESULTS OF SENSITIZATION TESTS IN THE GUINEA PIG 66

TABLE OF CONTENTS (Continued)

Page

5.0 HUMAN SKIN SENSITIZATION TEST PROCEDURES 74
5.1 Schwartz and Schwartz-Peck Test 74
5.2 Shelanski-Shelanski Test 74
5.3 Modified Draize Test 74
5.4 Maximization Test 77

6.0 APPLICATION OF PREDICTIVE TESTS TO KNOWN CONTACT ALLERGENS . 79
6.1 Human Tests 79
6.2 Guinea Pig Tests 79

7.0 USE OF HUMAN TESTS 84

8.0 CONCLUSIONS 87

9.0 RECOMMENDATIONS 88

10.0 REFERENCES 89

TABLES

2-1 Predictive Tests for Guinea Pig Skin Sensitization . . . 53

2-2 Comparative Results of Sensitization Test in Guinea Pig 68

2-3 Percent of Animals Reacting per Group in the Optimization, Maximization and Epidermal Test 70

2-4 Comparison of Six Protocols for Development of Delayed Hypersensitivity to Seven Compounds 71

2-5 Allergenicity of 32 Incriminated Compounds for Humans and Determinations of Their Allergenicity in the Guinea Pig by Four Test Methods 73

2-6 Predictive Patch Tests for Human Skin Sensitization . . 75

2-7 Predictive Patch Test in Humans 80

2-8 Reproducibility of Maximization Test Grades in Humans . 81

2-9 Grades of Allergenic Potency by the Maximization Tests in Humans and Guinea Pigs 82

2-10 Sensitization Rate in Guinea Pig Closed Patch Test and the Human Repeated Insult Patch Test 83

2-11 Evaluation of Some Common Sensitizers by the Diagnostic and Predictive Tests 85

TABLE OF CONTENTS (Continued)

Page

PHOTOTOXICITY

1.0 SUMMARY 93

2.0 PHOTOSENSITIVITY 94

3.0 PHOTOTOXICITY TESTING 97
3.1 Animal Models 97
3.2 Human Testing 97

4.0 PHOTOALLERGY TESTING 101
4.1 Animal Models 101
4.2 Human Testing 101

5.0 CONCLUSIONS 103

6.0 REFERENCES 104

TABLES

3-1 Comparison of Phototoxic and Photoimmunologic Reaction 95
3-2 Phototoxic and Photoallergic Chemicals 96
3-3 Phototoxicity Testing in Animals 98
3-4 Phototoxicity Testing in Humans. 100
3-5 Photoallergy Testing in Guinea Pig 102

SYSTEMIC DERMAL TOXICITY

1.0 SUMMARY 105

2.0 INTRODUCTION 106

3.0 FACTORS INFLUENCING ABSORPTION AND TOXICITY 107
3.1 Properties of Test Agents 107
3.2 Species Differences 107
3.2.1 Recommendations 109
3.3 Regional Variation in Absorption. 110
3.4 _In Vitro_ vs _In Vivo_ Absorption. 110
3.5 Use of Abrasion 111
3.6 Dermal LD50 Vs Oral LD50 112

4.0 ACUTE DERMAL TOXICITY TESTING 113
4.1 Duration of Exposure 113

5.0 LONGER TERM DERMAL TOXICITY TESTING 117

6.0 EXAMINATION 124
6.1 Irritation Assessment 124

TABLE OF CONTENTS (Continued)

Page

6.2 Histopathology and Clinical Measurements 124
6.2.1 Clinical Chemistry 124
6.2.2 Hematology 126
6.2.3 Recommendations 126

7.0 PUBLIC COMMENTS 128

8.0 CONCLUSIONS 129

9.0 REFERENCES 131

APPENDIX

Comparison of Oral and Dermal Lethality 134

TABLES

4-1 Acute Dermal LD50 of Test Compounds in the Rabbit and Guinea Pig 109

4-2 Effect of Anatomic Region on Absorption of Pesticides in Humans 110

4-3 Absorption in Human Skin 111

4-4 Relationship Between Toxicity and Safety of Pesticides . 112

4-5 Comparison of Guidelines for Acute Dermal Toxicity . . . 114

4-6 Comparison of Guidelines for Subchronic Dermal Toxicity 119

FIGURE

4-1 Absorption Variation by Species. 108

PREFACE

The Environmental Protection Agency (EPA) is formulating a uniform set of toxicity testing guidelines based on those recommended by the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the Toxic Substances Control Act (TSCA), the Interagency Regulatory Liaison Group (IRLG), and the Organization for Economic Cooperation and Development (OECD). In support of this activity, EPA's Office of Pesticides and Toxic Substances has requested Tracor Jitco to critically review, summarize, and evaluate the available information on dermatotoxicity testing. Tracor Jitco has examined dermal irritation, sensitization, phototoxicity, and systemic toxicity. These testing procedures were reviewed and assessed in terms of scientific rationality, effective use of animal resources, reliability in predicting effects in humans, practicality, and cost-effectiveness.

To evaluate the various protocols used in dermatotoxicity studies for approximately the last 20 years, literature searches were made using computerized data bases and published indexes. Relevant articles published earlier were manually retrieved by "tree searching" of references cited in the original article. Data bases searched included: Toxline and Medline, BIOSIS, SCISEARCH, CHEMICAL ABSTRACTS, EXCERPTA Medica, NIOSHTIC, NTIS and RTECS. Information was also obtained from laboratories engaged in dermatotoxicity testing and from recognized experts.

After receiving this information a critical review was performed by Tracor Jitco toxicologists. The next step in the process was to evaluate the literature and public comments relative to the specific EPA guidelines. For example, there is disagreement on the best choice of species for particular studies. More than one species is justifiable in many cases. Other topics requiring additional refinement included: the use of abraded skin, the patch test methods of scoring, degree of occlusion, clinical chemistry measurements and histopathological studies. When possible an attempt was made to compare and contrast the accepted guidelines with other available information to arrive at an independent recommendation or conclusion.

The data obtained from the literature survey are organized into four major sections in this report: the first section deals with Dermal Irritation, the second with Dermal Sensitization, the third with Dermal Phototoxicity and the fourth with Systemic Dermal Toxicity.

Each section is organized as a separate unit complete with information on the objective and scope of the survey, a description of the pertinent findings, tables and figures, conclusions and/or recommendations and a summary.

This report has been reviewed by individuals within the EPA, but has not been externally peer reviewed. The authors would like to thank James Murphy, Cheryl Peterson, and Mark Townsend of the EPA for their technical review and helpful suggestions.

EXECUTIVE SUMMARY

A major objective of toxicity testing guidelines is to ensure the gathering of data which can be used to reliably predict effects in humans prior to exposure of the general population to potentially hazardous substances. Guideline tests should be cost-effective, specific, and designed so as to minimize the numbers of animals used and the trauma to each animal.

This report examines the four categories of Dermatotoxicity testing: Dermal Irritation, Dermal Sensitization, and Systemic Toxicity, all of which presently have testing guidelines, and Phototoxicity, for which guidelines are currently being developed.

In conducting this assessment, a number of areas and issues were considered in detail. These include species selection, testing procedures/protocols, sensitivity and enhancement of reaction to test agents, predictability of human response from animal data, and test result evaluation.

Dermal Irritation

Although dermal irritation studies historically, have been widely conducted on rabbits, the findings of this report suggest that the guinea pig should also be considered as an acceptable animal model, as the sensitivity of this species is comparable to that of the rabbit for many compounds.

Other species of animals have also been used in dermal irritation studies. These include the minature swine, rat, mouse, piglet, beagle, baboon, hairless rat, hairless hamster, and hairless mouse. In addition to reliably detecting irritancy, species selection criteria should include practical considerations. For example, the monkey, is rarely used because of cost and non-availability, even though its response mimics that of the human.

Factors affecting the results of dermal irritation studies include: 1) the patch test unit, 2) the degree of occlusion, 3) use of abrasion, 4) application of the test substance, 5) the application site, 6) the duration of exposure and observation and 7) the techniques used in evaluating the test results.

The simple gauze patch is the most commonly used technique. The use of an occlusive chamber that ensures reproducibility in the amount of substance applied to a given area of skin is a recent development that deserves further evaluation. In the standard patch test, present guidelines allow for varying the degree of occlusion, the application site, and the method of moistening a solid substance. Nevertheless, a need may exist to control these variables. On the other hand, the use of abrasion and the length of exposure and the observation period are factors that generally are rigidly defined. Greater flexibility in these criteria may, however, permit the development of more information on a compound. While increased flexibility will allow the investigator to enhance the sensitivity of the test to suit conditions of use and population exposure, constancy in the length of the exposure and observation periods contributes to comparability of test results with different compounds.

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Variability in the results of dermal irritation studies can result from the methods used for evaluation or scoring. The development of a reference set of photographs or slides depicting the various grades of irritation could facilitate the evaluation by ensuring consistent grading, terminology usage, and scoring. A possible alternative scoring method that should be considered is the use of a quantal response, i.e., the IT50 (number of days before noticeable irritation occurs in 50% of test animals) or the ID50 (the concentration that produces noticeable irritation in 50% of the test animals).

The data evaluated in this review suggest that the use of a tier-like strategy may be an effective means of obtaining information on the dermal irritation potential of test substances. As a preliminary step, test materials may be measured for pH. According to the OECD guidelines, substances with a pH of 2 or less or 11.5 or greater do not need to be tested for dermal irritation based on the assumption that they will be irritating. Where substances are not screened out by pH determinations, the hairless mouse could be used as a test species for the prescreening of irritancy. Further characterization of irritancy could be obtained by testing in the rabbit or guinea pig. Depending on anticipated use and exposure hazards of the test materials, human subjects could then be used to test compounds of low or marginal irritancy.

Dermal Sensitization

Dermal sensitization can be defined as the delayed immune response to an antigenic substance applied to the skin. The guinea pig is the species most widely used because it is generally effective in determining the sensitization potential of a chemical. In certain cases, however, this test species has not demonstrated the subtle effects observed in humans. The OECD lists eight guinea pig methods which it considers acceptable. These can be divided into three groups depending on whether the route of administration is intradermal, epicutaneous or a combination of the two. These tests involve an initial exposure to the test material (induction) followed by a rest period of approximately 2 weeks and a second exposure (challenge). A positive challenge reaction indicates sensitivity, assuming that the control group shows no response. The Maximization and Closed Patch tests are currently the most widely used, although no one method has been sufficiently validated to support its selection as the method of choice. The Maximization test provides more reliable detection of weak sensitizers than the earlier Draize method. Based on a small amount of published data, the Optimization and Open Epicutaneous tests also appear effective in providing information on sensitization responses to test agents.

In testing regimens requiring exact prediction of response in a large human population (i.e., allergy testing), the use of human subjects is a common practice. In all instances, prescreening of substances in guinea pigs is necessary prior to testing in humans. The Maximization test in humans most accurately predicts the response to the general population. Use of this test to validate guinea pig test methods has demonstrated good quantitative agreement between the response of the guinea pig and man to a variety of test chemicals.

A satisfactory skin sensitization program for chemicals, pesticides, drugs, paints and coatings, toiletries and cosmetics may involve both the guinea pig and man. Positive (strong sensitization) results in the guinea pig preclude the testing on humans; a weak or negative response may indicate the need for further characterization and carefully controlled testing in small groups of human subjects. For the latter case, the Maximization test should be used.

Phototoxicity

Ultraviolet light can alter a chemical that normally produces no toxic responses into one causing direct toxicity or allergenicity. With the exception of fragrance ingredients, only a small number of chemicals have been extensively studied for phototoxic potential. Light activation of the test substance can elicit either a toxic or sensitivity response. The guinea pig, hairless mouse and minature swine have been used to study phototoxicity, but present studies do not permit evaluation of the most appropriate species for testing.

In methods that have been used for phototoxicity studies, the test agent is either applied topically to the skin of the test animal or delivered by intraperitoneal injection. After a specified time interval, the test site is irradiated with ultraviolet light and reactions are scored. The OECD is currently developing guidelines in this area.

Systemic Toxicity

Dermal toxicity testing is done to determine whether a substance can be absorbed in quantities sufficient to produce systemic effects, as well as the nature of such effects. Some of the factors that influence the degree of irritation produced by an agent will also influence its systemic toxicity. These include characteristics of test agents such as pH and lipid/water solubility and specific test procedures, such as abrasion of the skin and the methods used to apply test substances. A dermal study alone will rarely be sufficient to completely characterize the toxic effects of an agent, as it provides information on the effects produced by only one route of exposure. The OECD guidelines suggest testing the rat, rabbit or guinea pig. With the IRLG guidelines, preference is given to the rabbit. The data evaluated indicate that the rat is a more appropriate species to study systemic effects after dermal exposure. This is primarily because much of the available toxicity data resulting from tests by other routes of exposure have been obtained from the rat. If LD50 values from different routes are compared in the rat, the relative rate of percutaneous absorption of a series of compounds can be estimated.

A comparison of LD50 values for rabbits and rats shows that, in more than 75% of the documented cases, the LD50's varied by less than a factor of four, with neither species clearly showing greater sensitivity. It has also been shown that the LD50 values were similar whether a rabbit was used for 24 hours or a rat was used for 4 hours.

Dermal toxicity studies of longer duration are limited in their practicality and cost-effectiveness. Animal restraint, patch attachment and laboratory personnel are necessary for long periods. In addition, systemic effects can be adequately determined by administration of the agent by a more cost-effective route. Likewise, extensive clinical chemistry measurements and histopathology studies should be selectively performed, depending on the intended use of the substance. Inclusion of these additional measurements can be appropriate, however, in tests carried out by other routes.

When dermal toxicity studies are performed, particular attention should be paid to the size of the patch, the area of skin in relation to the size of the animal, the degree of occlusion, and the concentration and amount of test substances to allow development of consistent data.

UNRESOLVED ISSUES AND RESEARCH RECOMMENDATIONS

Dermal Irritation

- Further evaluation of guinea pig, rabbit and hairless mouse
- Validation of occlusive chambers in animals
- Validation of IT50 and ID50 in animals
- Preparation of an illustrated guide for the grading of dermal irritation
- Further studies to determine the optimum exposure period and degree of occlusion for industrial chemicals

Sensitization

- Extensive cross-validation of the eight guinea pig test methodologies with a variety of industrial and other chemicals

Phototoxicity

- Further examination to determine the appropriate animal model for phototoxicity testing

Systemic Toxicity

- Determination of the appropriateness of the rabbit, guinea pig and rat for long-term studies
- Establishment of minimum and optimum exposure periods in acute testing

DERMAL IRRITATION

1.0 SUMMARY

A major goal of testing substances for dermal irritation is reliability in predicting the human response. All guidelines, with the exception of the National Academy of Sciences (1977), recommend the use of the albino rabbit as the test species. The rabbit is primarily used because it is readily available, easy to handle, and as it has been preferred historically, use of the rabbit allows comparison of results with a large number of test substances in the same species.

Humans come into contact with a myriad of chemicals such as solvents, industrial compounds, pesticides, detergents, household chemicals, and cosmetic products. The chemical industry is interested primarily in the effects of short-term exposure from spills and more prolonged exposure from normal usage. Tests attempting to predict the expected effects in humans should make allowances for possible delays in decontaminating clothing and skin and for the protection of individuals who show a much greater sensitivity than the normal population. The cosmetic and toiletries industries are most interested in preventing even mild irritation that might occur in a small fraction of exposed consumers. These industries use the direct approach through paid volunteer test subjects, avoiding the uncertainty of interspecies extrapolation and providing adequate prediction of human irritation responses. Results of several studies support the use of the guinea pig because of practical considerations and its ability to predict the human response. The hairless mouse has also been studied to a limited extent. In actuality, the choice of test species should be based on practical considerations, cost-effectiveness, and on the ability to detect irritation effects from expected exposures.

The Federal Hazardous Substances Act, in 16 CFR 1500.4, stipulates that human data should be taken into account in evaluating a substance. Studies in humans are generally performed to characterize possible responses to commercial products and substances that are intended for use on human skin or present other special exposure hazards. Most human studies employ patch testing procedures with modifications to detect irritation effects under conditions of anticipated product use and exposure.

2.0 INTRODUCTION

2.1 Objective

The objective of this survey is to investigate the factors affecting the predictability of dermal irritation studies and the reproducibility of their results, in particular with reference to the OECD (1981) guidelines. The main purpose for measuring dermal irritation is to differentiate between substances that cause moderate or strong irritation or corrosion of the skin and those compounds which do not. Three major areas of concern are addressed in the following pages: selection of species for testing, testing procedures, and evaluation of results. In some cases modifications of the current guidelines have been suggested when appropriate.

2.2 Definitions

Irritation is the local inflammatory response of the skin to direct injury by a single, repeated, or prolonged contact with a chemical without the involvement of an immunological mechanism. Erythema and edema are the macroscopic manifestations of irritation. Corrosion (the correct pathological term is erosion) is the direct chemical action on normal living skin which results in its disintegration and irreversible alteration at the site of contact. Corrosive chemicals cause ulceration, necrosis, fissuring, and with time, the formation of scar tissue.

2.3 Historical

One of the earliest descriptions of the use of the patch test for dermal testing was provided by Fabre in 1898. After handling a caterpillar and suffering a reaction, Fabre tested an extract of the insect's hair and described his procedure as follows:

"When the etheral infusion is reduced by evaporation to a few drops, I soak a slip of blotting-paper folded in four, so as to form a square measuring something over an inch...Lastly, the square of paper,...is applied to the under surface of the forearm. A thin water-proof sheeting covers it, to prevent it from drying too rapidly; and a bandage holds it in place."

The bandage was kept in place for ten hours during which time nothing happened. Fabre then developed an acute eczematous reaction.

"I experience an increasing itch and a burning sensation acute enough to keep me awake for the greater part of the night. Next day, after 24 hours of contact, the poultice is removed. A red mark, slightly swollen and very clearly outlined, occupies the square which the poisoned paper covered. The skin feels sore, as though it had been cauterized, and looks as rough as shagreen. From each of its tiny pustules trickles a drop of serous fluid, which hardens into a substance similar in color to gum arabic. This oozing continues for a couple of days or more. Then the inflamation abates; the pain, hitherto very trying, quiets down, the skin dries and comes off in little flakes. All is over, except the red mark, which remains for a long time, so tenacious in its effects is the extract of Processionary (the caterpillar genus). Three weeks after the experiment, the little square on the forearm subjected to the poison is still discolored."

Many of the practices used by Fabre, including the inch square patch on the inside of the forearm and the occlusive covering held in place for several hours foreshadowed the general approach to dermal irritation testing developed in the years that followed.

The original Draize test called for a patch test on previously clipped albino rabbits using both intact and abraded skin sites. Abrasions were defined as minor incisions through the stratum corneum that did not reach the dermis and induce bleeding (Draize, 1955). The test chemical was introduced under gauze patches applied to the backs of albino rabbits, which were clipped free of hair. Applications were made on three rabbits with intact skin and three with abraded skin. The patches were secured by adhesive tape, and the entire trunk of the animal was wrapped in rubberized cloth for 24 hours. The animals were immobilized during the exposure period, the patches were removed after 24 hours, and the resulting reaction evaluated visually for erythema and edema according to the scoring system shown in Table 1-1. The reaction was also scored after 72 hours. A Primary Irritation Index (PII) was obtained by averaging the 24- and 72-hour reaction scores. The PII was then used to rate the chemical as a non-irritant, a moderate irritant or a severe irritant (Table 1-2). The Draize method is summarized in Table 1-3.

In 1959, the procedure was finalized with the addition of the step of wetting solids to be tested with an appropriate solvent prior to testing. The procedure was published in the Food, Drug, and Cosmetic Law Journal. The Draize procedure was adopted by the Food and Drug Administration as the official test for primary skin irritation and has since been legislated under provisions of the Federal Hazardous Substances Act (FHSA) (29 CFR 13009 191.11) (Table 1-3). The Consumer Product Safety Act (CPSA) (15 U.S.C. 2051; 1972) vested authority for the FHSA to the Consumer Product Safety Commission (CPSC) for determining hazard labelling. Presently, the method of testing primary irritant substance can be found at 16 CFR 1500.41. Since evaluation of irritation threshold is necessary for labelling a product, FHSA states that a test agent is considered to be a primary irritant if it scores 5 or higher in the test (see Table 1-2). Further adaptations of the Draize procedure have deemphasized this cut off for primary irritation versus non-irritation due to reported variability both in absolute scoring of the standard FHSA protocol as well as rank ordering of irritation of compounds (Weil and Scala, 1971).

In 1972, the FDA reported a revision of the test procedures that reduced the exposure period to 4 hours and required the detailed evaluation of the corrosive effects due to a concern "that the skin irritation test does not realistically reflect the skin contact that could reasonably be expected from exposure,... (as)...immediate steps would probably be taken to flush or remove a substance when irritation is perceived". This proposal, excluding the requirements for testing substances on abraded skin, has been adopted by the Department of Transportation for classifying corrosive substances (49 CFR 173.1200 Appendix A).

Table 1-1. Evaluation of Skin Reactions: Single Application

A. Erythema and eschar formation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness to slight eschar formation-injuries in depth)	4
Total possible erythema (or score)	4
B. Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (area raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond area of exposure	4
Total possible score	4
Total possible score for primary irritation (Primary Irritation Index, PII)	8

[a] (Draize et al. 1944)

Table 1-2. Primary Irritant Response Categories in the Rabbit (Draize et al., 1944)

Response Category	Mean Score (Primary Irritation Index - PII)
Negligible	0 - 0.4
Slight	0.5 - 1.9
Moderate	2 - 4.9
Strong (Primary Irritant)	5 - 8.0

Table 1-3. Comparison of Methods Used for Dermal Irritation

	O.E.C.D.	I.R.L.G.	DRAIZE	N.A.S.(1977)[a]	D.O.T.
SPECIES	ALBINO RABBIT	SAME[b]	SAME	ALBINO GUINEA PIG PREFERRED, RABBIT	ALBINO RABBIT
SEX	N.S.[c]	N.S.	N.S.	N.S.	N.S.
AGE	ADULT	SAME	N.S.	YOUNG ADULT	N.S.
WEIGHT	N.S.	2-3 Kg	N.S.	N.S.	N.S.
NUMBER	3 minimum	6 minimum	6	6	6 minimum
		3 for limit test plus 3 additional if results equivocal			
NUMBER OF PATCHES PER COMPOUND	N.S.	1	4	N.S.	N.S.
DOSE	0.5 ml undiluted liquid or	SAME	SAME undiluted	SAME	SAME
	0.5 gm solid or semi-solid	SAME but premoisten sample and gauze with water or solvent	SAME but dissolv solids in appropriate solvent	SAME but moisten with solvent (50% slurry)	SAME as OECD
PATCH SIZE	6 cm^2	SAME	1 in^2	1 in^2	1 in^2
PATCH MATERIAL	Gauze Patch	SAME	SAME - 2 layers thick	GAUZE or other inert semi-absorbant material i.e. 2- or 12-ply gauze, non-woven cotton fabric or cellulose pads	GAUZE, 2-ply
PATCH TAPE AND COVER	Nonirritating tape loosely held by semiocclusive dressing	Nonirritating tape loosely held using impermeable material (rubberized cloth or plastic fiber)	Adhesive tape - entire trunk wrapped with impervious material (rubberized cloth)	porous tape with rubberized cloth or stockinetta	SAME as Draize
	Occlusive dressing may be used	Not recommended unless human exposure warrants	N.S.	N.S.	Do not occlude
	Access to patch should be prevented	Immobilized animals in stocks or Newman Harness	N.S.	Collars or restraint	N.S.

Table 1-3. Comparison of Methods Used for Dermal Irritation

(Continued)

	O.E.C.D.	I.R.L.G.	DRAIZE	N.A.S.(1977)	D.O.T.
EXPOSURE	4 hours	4 hours	24 hours	4 hours	4 hours
WASHING	Allowed	N.S.	N.S.	N.S.	Allowed
ABRASION	Not required	Not required	Abrasion + normal skin for each animal	Not recommended	Not required
EXAMINATION	1/2 to 1, 24, 48, and 72 hrs. after patch removal	SAME	Read after 24 hr. exposure and 48 hrs. later	SAME except no reading at 48 hrs.	After 4 hours and at 48 hours
SCORING	Draize Scoring	SAME, report average of all values at all times of observation	SAME Add erythema and edema values for abraded and intact skin at both times and divide by four	SAME Add erythema and edema scores at each time, use highest mean score	Evaluate corrosion only
CORROSION	Note if corrosive	Note if corrosive	Not considered	Not considered	Note if corrosive
MISCELLANEOUS	Further observation if needed, Record previous lesions/toxic effects Describe degree and nature of irritation	Possibly use histopathology		Corrosion test seen at 7 days	

[a] from National Academy of Sciences (1977)
[b] Same as OECD
[c] Not Specified

The Federal Insecticide, Fungicide and Rodenticide Act (FIFRA - 40 CFR 162) requires that pesticides be labeled with toxicity categories (from I to IV). The degree of dermal irritation at 72 hours is one of the criteria for assignment to a toxicity category. In June 1975 and August 1978, the EPA published proposed FIFRA test guidelines for measurement of dermal irritation. TSCA guidelines were proposed in May and June of 1979 (40 FR 26802, 43 FR 37336, 44 FR 27334 and 44 FR 44054). Due to differences in wording and approach between FIFRA and TSCA test guidelines, the more broadly based Interagency Regulatory Liaison Group (IRLG) and later the Organization for Economic Cooperation and Development (OECD) guidelines were adopted. These two methodologies, along with those of the FHSA (CPSC), DOT and the National Academy of Sciences (1977), are compared in Table 1-3. Differences in the guidelines are discussed in the following sections. The OECD guidelines have taken into account and addressed many of the scientific issues which have arisen from public comment on the FIFRA and TSCA guidelines. Corrosivity is to be noted as part of the OECD reporting procedure and is specifically mentioned in the section on Minimum Premarket Data of the TSCA guidelines but scoring of corrosion per se is not required. Adoption of this procedure will allow OECD guidelines to fulfill DOT Regulations.

Friedman, in his General Referee Report on Toxicological Testing to the AOAC commented: "Toxicological testing procedures have, until recently, not been considered suitable subjects for standardization. The determination of whether a given material has the capability of evoking a biological response is usually a problem of such complexity that it does not lend itself to standardization. There are stages, however, in the development of any problem. In the beginning there is a need for a maximum of innovation and creativity in devising the approaches and techniques that might be useful. In the case of a complex problem, this stage where standardization is contra-indicated may last a long time...The danger of standardization lies in the possibility of fixing systematic errors into a procedure and achieving a desired degree of reproducibility at the price of accuracy and innovation," (Goldberg, 1975). Some testing procedures to be discussed are still in the developmental stage; others are well established. They are analyzed here in light of new scientific discoveries and thinking over the last two or three decades.

2.4 pH of Test Agents

The design of a skin irritation test should take into account the physical and chemical properties of the compound. According to the OECD guidelines, substances with a pH of 2 or less or 11.5 or greater do not need to be tested for dermal irritation based on the assumption that they will be highly irritating to the skin.

Guillot and coworkers (1981) studied the effect of pH on the primary cutaneous irritation responses produced by a variety of substances. Measurements of pH were performed on the test agents; the pH of solid materials was measured from a saturated solution of the pulverized substance in distilled water. Substances were tested on the clipped skin surface of the scarified right flank and on the intact left flank of albino rabbits. Liquid test agents were applied to gauze pads and occluded to the application site. Solid substances were pulverized, applied to the site, covered with a gauze pad that was moistened with water, and occluded to the skin. Of highly acidic substances, dimethylsulfate, a liquid with a pH of 1.0, was severely irritating, while two solid substances, aluminum nitrate, pH 0.8; and oxalic acid, pH 1.0, were non-irritating and moderately irritating, respectively. Four solid substances with pH values between 2.1 and 2.7 were all non-irritating. Of highly alkaline substances, one solid with a pH of 10.7 (scouring powder) was non-irritating, while four liquids showed the following pH values and corresponding results: pH 10.1, non-irritating; pH 10.8, slightly irritating; pH 10.5 and 10.7, moderately irritating. The above results show that solid acidic substances in solution with a pH of 2 or less cannot be assumed to be severely irritating. The apparent discrepancy between the results of highly acidic liquids and solids may be explained by the different properties (e.g., wetting properties) of these substances as tested. In addition, further studies or additional data would be useful to more fully validate the OECD test exclusion for highly alkaline substances. The OECD exemption is a reasonable one, but it does not prohibit further testing if indicated by a need to know more about either a specific chemical or the physicochemical properties that enhance or retard irritancy.

3.0 SPECIES SELECTION

3.1 Animal Tests

The use of the rabbit as the preferred test species is evidenced by the large amount of dermal irritation information on this species in the Registry of Toxic Effects of Chemical Substances (RTECS). Eighty-five percent of over 2,000 RTECS entries report test results with the rabbit, 7.5% with the human, 4% with the mouse and 3% with the guinea pig.

The majority of the human data comes from the Research Institute for Fragrance Materials Monographs on essential oils and other aromatics published in Food and Cosmetic Toxicology. This is also the primary source of mouse skin irritation data.

The choice of proper species for measuring dermal irritation and predicting the effects of chemicals on humans has been actively discussed since its requirement in the Federal Hazardous Substances Labeling Act in 1961. The OECD and IRLG guidelines both specify the use of the albino rabbit. The Department of Transportation also requires the use of rabbits in the identification of corrosive substances (49 CFR 173.1200 Appendix A). As a result, rabbits have been used to generate a vast majority of the available data in the open literature. Despite the wide use of rabbits, the National Academy of Sciences (1977), suggests that the guinea pig and not the rabbit be used as the preferred test animal based on its response being more like that of human skin over a wide range of materials and the more economical requirements for space and caging.

3.2 Interspecies Comparison

Roudabush et al. (1965) compared the acute effects of 14 chemicals on the skin of rabbits and guinea pigs using procedures described in 21 CFR 191.11 (Federal Hazardous Substances Act). Their results indicated that the responses of these two species are essentially equivalent when scores for intact and abraded skin were averaged. In the guinea pig, scores were higher compared to rabbit on intact skin and lower on abraded skin (Table 1-4). Household ammonia and trisodium phosphate acted as primary irritants only in the guinea pig. Accordingly, and in consideration of cost and space requirements, the authors concluded that there is sound justification for using the guinea pig as an alternative species to the rabbit for skin irritation studies.

The rabbit is not a good model for moderately and minimally irritating materials (NAS, 1977) as these substances often show stronger response in the rabbit than in humans. It does however, correctly identify compounds which are highly irritating to humans, thus avoiding unnecessary risk in exposing the volunteer test force to these compounds.

In attempting to more accurately predict human response, other animals have been tested with mixed results. These include the guinea pig, miniature swine, rat, piglet, beagle, baboon, hairless rat, hairless hamster and hairless mouse. The guinea pig and the hairless mouse have been used more often than any of the others.

Table 1-4. Summary of Primary Irritation Scores in Rabbit and Guinea Pig[a]

Compound	Animal	Intact Skin	Abraded Skin	Average[b]
Acetic acid, 4% aqueous	Rabbit	1.5	3.6	2.6
	Guinea pig	1.9	1.9	1.9
Ammonia, household	Rabbit	3.2	5.0	4.1
	Guinea pig	5.6	5.0	5.3
Borax	Rabbit	1.6	2.3	2.0
	Guinea pig	1.5	1.4	1.4
Boric acid	Rabbit	0.8	2.5	1.7
	Guinea pig	2.7	1.4	2.1
2-Butanone	Rabbit	0.8	2.7	1.8
	Guinea pig	1.6	2.4	2.0
Carbon tetrachloride	Rabbit	2.8	4.0	3.4
	Guinea pig	4.1	2.2	3.1
Gasoline	Rabbit	2.0	2.9	2.4
	Guinea pig	3.9	2.8	3.3
Kerosene	Rabbit	1.9	3.2	2.5
	Guinea pig	3.6	2.6	3.1
Phenylhydrazine	Rabbit	1.8	3.0	2.4
	Guinea pig	3.6	2.6	3.1
Sodium carbonate	Rabbit	2.1	5.0	3.5
	Guinea pig	2.1	2.6	2.3
Sodium sulfite	Rabbit	1.6	3.1	2.4
	Guinea pig	2.4	1.7	2.0
Sucrose	Rabbit	0.4	2.5	1.4
	Guinea pig	1.5	0.9	1.2
Trisodium phosphate	Rabbit	3.4	5.1	4.2
	Guinea pig	6.3	5.1	5.7
Water, distilled	Rabbit	1.1	1.8	1.4
	Guinea pig	1.3	1.0	1.1
Range	Rabbit	0.4-3.4	1.8-5.1	1.4-4.2
	Guinea pig	1.3-6.3	0.9-5.1	1.1-5.7

Table 1-4. Summary of Primary Irritation Scores in Rabbit and Guinea Pig[a]

(Continued)

Compound	Animal	Intact Skin	Abraded Skin	Average[b]
Average	Rabbit	1.79	3.34	2.56
	Guinea pig	2.87	2.40	2.69
Correlation coefficient to rabbit, intact plus abraded	Rabbit	0.943	0.962	-
	Guinea pig	0.803	0.861	0.847

[a] Adapted from Roudabush et al (1965)

[b] The calculations of this primary irritation score is described in 21CFR 191.11. In the other columns for intact or abraded skin, the sum of the values at 24 and 72 hours was divided by 2 rather than 4.

Davies et al. (1972) found considerable interspecies variation in dermal irritation, as summarized in Table 1-5. The major interest of these authors was the screening of cosmetic ingredients, where false positives are acceptable but false negatives are not. Therefore, they felt that a test species should not be chosen based on its resemblance to man but based on which is the most sensitive, namely the rabbit and guinea pig. Testing in the dog and miniature swine give false negative results for cream shampoo, propylene glycol, aluminum chlorohydrate, and sodium lauryl sulfate.

Brown (1971) compared the irritant effects of surfactants (labeled T1 through T9) on humans, rabbits, guinea pigs and hairless mice without occlusion. When the gross and histological irritancy is graphed on an arbitrary scale of 1 (least irritating) to 5 (most irritating), the rabbit and the guinea pig give equivalent responses which are reasonably predictive of human response giving only false negatives for T-9. The hairless mouse was less accurate, giving a false negative score for T-9 and a higher reading for T-1. The results are depicted in Table 1-6.

Marzulli and Maibach (1975) compared the results of a 16-day cumulative irritation test in rabbits (uncovered test site) with those from a 21-day test in man (covered test site) using antimicrobials, sunscreens, acids and alkalis, detergents, anti-perspirants, vitamin E preparations, cosmetics, and an antipsoriatic. Results for 60 test materials showed a significant correlation ($r = 0.30$; P less than 0.02) between the scores obtained for rabbits and man. In most cases, the cumulative scores were higher for rabbits, indicating a greater sensitivity. However, oxalic acid and sulisobenzone gave higher scores in man. The authors concluded that repeated application serves as a valuable modification to the Draize test and that the greater responsiveness of the rabbit offers a useful margin of safety for prediction of irritation in man.

Griffith and Buehler (1977) compared skin response in humans with rabbits and guinea pigs using a patch test. Twenty-four chemicals and household products were tested and scored. Highly lipophilic compounds (soaps and detergents) showed the greatest tendency toward exaggerated response in rabbits. Both species gave 16 "correct" results when compared with man, but tended to err on the high side with "weak" irritants. The results for the guinea pig (intact skin) were more often on the low side for slight or moderate human skin irritants, as shown in Table 1-7. It should be noted that for industrial chemicals (Table 1-4) the guinea pig scored higher in general, however, ammonia tested by each group gave opposite results.

Tests for primary irritation using the albino mouse ear skin (Uttley and Van Abbe, 1973) (Table 1-8) and the back of the hairless mouse (Brown, 1971; Kastner, 1977; and Homberger et al., 1962) have been suggested as preliminary screening procedures before initiating patch testing in man. The sensitivity observed in 24-hour patch tests using the hairless mouse has more closely resembled human response to over 60 compounds than that observed for either the rabbit or guinea pig (Kastner, 1977). The compounds tested included fatty acids, alcohols, amides, detergents, esters, and cosmetic bases. Hairless mice have also proven to be useful in the testing of marketed sunscreening preparations (Gloxhuber, 1976).

Table 1-5. Relative Irritancy, Man vs. Seven Other Species[a]

Species	Relative irritancy		
	Similar to Man	More than Man	Less than Man
Mouse	Lanolin	Sodium lauryl sulphate Aluminium chlorhydrate Para-phenylenediamine	Propylene glycol (1% and 5%) Cream shampoo Thioglycolate paste
Guinea pig	Lanolin	Thioglycolate paste Aluminum chlorhydrate Sodium lauryl sulphate (1% and 5%) Para-phenylenediamine	Propylene glycol Cream shampoo
Rabbit	Lanolin	Thioglycolate paste Propylene glycol Aluminium chlorhydrate Sodium lauryl sulphate (1%)	Sodium lauryl sulphate (5%) Para-phenylenediamine Cream shampoo
Mini pig	Lanolin Thioglycolate paste Sodium lauryl sulphate (5%) Para-phenylenediamine		Propylene glycol Aluminum chlorhydrate Sodium lauryl sulphate (1%) Cream shampoo
Piglet	Propylene glycol	Lanolin Thioglycolate paste	Cream shampoo Aluminium chlorhydrate Sodium lauryl sulphate (1% and 5%) Para-phenylenediamine
Dog	Lanolin Aluminium chlorhydrate Sodium lauryl sulphate (5%) Para-phenylenediamine	Cream shampoo	Propylene glycol Sodium lauryl sulphate (1%)
Baboon	Lanolin Thioglycolate paste		Propylene glycol Aluminium chlorhydrate Cream shampoo Para-phenylenediamine Sodium lauryl sulphate

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[a] Adapted from Davies, et al. (1972)

Table 1-6. Ranking of Skin Irritation Results with Surfactants on Human and Animal Skin[a]

	Uncovered Exposures			
Irritancy Rating	Human Arm Immersion Tests	Rabbits	Guinea Pig	"Hairless" Mice
Least Irritant 1	T3 T6[b]	T1 T2 T3 T4 T5 T6 T9	T1 T2 T3 T4 T5 T6 T9	T2 T3 T6 T9
2	T1			
3	T5	T7	T7	T1 T5 T7
4	T9	T8	T8	T8 T4
Most Irritant 5	T8			
Not Tested	T2 T4 T7			

[a] Adapted from Brown (1971), (Gross and/or Histological Examination)
[b] Surfactants are labelled T1 through T9

Table 1-7. Irritation Response of Human, Guinea Pig, and Rabbit Skin to Chemicals/Household Products[a]

Material	Mean Score (intact skin)		
	Human	Guinea Pig	Rabbit
Isopropyl alcohol, 100%	0.0[b]	0.0	0.0
Table salt, 50%	0.0	0.0	0.0
Sodium tripolyphosphate 50%	0.0	0.0	0.0
Sodium carbonate, 50%	0.0	0.0	0.0
Sulfuric acid, 10%	0.2	0.0	0.0
Sodium lauryl sulfate, 50%	0.6	0.0	1.6
Acetic acid, 10%	1.0	0.0	0.0
C_8 - C_{10} fatty acid, 100%	2.3	0.1	4.4
Sodium metasilicates, 50%	—	1.7	cor[c]
Potassium hydroxide, 10%	—	cor[c]	cor[c]
Detergent granules:			
Low carbonate, 50%	0.0	0.1	0.7
High carbonate, 50%	0.0	0.0	0.9
Enzyme, 50%	0.0	0.0	1.3
Phosphate, 50%	0.0	0.2	1.2
Coconut oil soap, 50%	0.0	0.3	2.5
Antiperspirant, 100%	0.1	0.0	0.0
Liquid detergent, 100%	0.1	0.9	2.4
Lemon juice, 100%	0.2	0.0	0.0
Liquid cleaner, 100%	0.2	0.3	3.0
Liquid shampoo, 100%	0.9	2.1	3.7
Pine oil cleaner, 100%	1.0	2.5	3.6
Household ammonia, 100%	1.5	0.0	1.4
Metasilicate-carbonate detergent, 50%	3.0	0.0	cor[c]
Hypochlorite bleach, 100%	3.9	0.3	1.0

[a] Adapted from Griffith and Buehler (1977)

[b] Based on scale of increasing irritancy; mean score 0 to 8.0

[c] Corrosive.

Table 1-8. Assessment of Irritation -- Mouse Ear[a]

Reaction Category	Score
No visible blood vessels or erythema.	0
Few blood vessels, barely visible. No erythema.	2
Main blood vessels visible on lower half of ear. Slight erythema over lower third or base of ear.	4
Main blood vessels more obvious. Slight or generalized erythema.	6
Main blood vessels extended to edge of ear; capillary network extensively affected. Possible internal hemorrhage; erythema more pronounced; loss of suppleness of ear.	8
Pronounced blood vessels; extensive capillary network evident; marked erythema; possible frilling of ear margin	10
Pronounced blood vessels; extensive capillary network extending to ear margin; severe erythema; frilling and thickening of ear margin; crusting evident.	12
All of the above plus possible necroses with extensive crusting	14

[a] from Uttley and Van Abbe, 1973

In several reports from the Research Institute for Fragrance Materials published in Food and Cosmetic Toxicology, over 90 essential oils were studied in humans (48 hours, closed patch), rabbits (24 hours, occluded patch, intact and abraded skin), and mouse (several hours, open epicutaneous). Over 50% of the compounds were irritating in the rabbit but not in humans or the mouse; 17% of the compounds induced different responses in the human and the mouse and 28% were nonirritating in all three species. In three instances, the rabbit was predictive of the positive human response. Testing of the swine using 41 of the essential oils resulted in findings similar to those for the mouse, including, predicted nonirritation. Results of a limited number of tests using the guinea pig resembled those for the rabbit.

Analysis of the preceding studies indicates that the rabbit, guinea pig, and hairless mouse are useful in predicting irritant effects in man. However, as in any toxicity study, no one species will be a perfect model for all chemicals. The guinea pig, rather than being an alternative to the rabbit, may actually be complementary as some chemicals detected as irritants by one species are missed by the other. Other species that have not been tested as frequently may also be useful for detecting skin irritation in man, e.g. the hairless mouse. The objective is to discriminate quantitatively among mild irritants with tests that are simple, reproducible, inexpensive, and accurate. An approach to achieve this goal may be to use human volunteers with attention to first screening out the moderate and severe irritants through prior animal testing.

4.0 TESTING PROCEDURES

The current OECD guidelines evolved from the testing procedures developed by Draize (1955) (Table 1-3). According to the OECD method, the test chemical is introduced under gauze patches applied to the previously clipped backs of at least three albino rabbits. The patches are held in place by nonirritating tape for 4 hours, and the animals are restrained, preventing access to the patches. Reactions are evaluated visually for erythema and edema (Table 1-2) 0.5 to 1, 24, 48, and 72 hours after the patch is removed. A Primary Irritation Index (PII) obtained by averaging the 24- and 72-hour reaction scores is used to rate the chemical as a nonirritant, a moderate irritant, or a severe irritant (Table 1-2).

Modifications to commonly used testing methodologies have frequently been made to reduce the variability and/or increase the sensitivity of patch tests. Variations in testing procedures are outlined in Table 1-9. Major areas of concern include: 1) the patch test unit; 2) occlusion; 3) use of abrasion; 4) application of the test substance; 5) the application site; 6) the duration of exposure and observation; and 7) enhancement of visual scoring. Methodologies in humans and animals have been compared and studied for the usefulness of specific testing procedures and for the ability to reliably detect irritating substances.

4.1 Patch Test Unit

The accuracy and reproducibily of skin irritation tests depends on the method of applying and securing the test substance. The size, thickness, and patch material as well as the type of tape, volume of test material and the method of occlusion can all affect results. Although occlusion generally exaggerates the type of exposure that humans usually encounter, it must be remembered that the purpose of the test is to detect potential irritants and not necessarily to duplicate the response in man. Most procedures described in the literature involve use of adhesive tape to secure the patch, but little attention is given to the tightness of the fit. This can affect the degree of occlusion.

According to Frosch and Kligman (1976, 1979), the standard patch test has many flaws. Uniform exposure is hard to attain, reproducibility and accuracy are difficult because the test substance can escape, and, more importantly, there is a lack of sensitivity in detecting mild irritants. Recently much attention has been given to the use of an occlusive chamber in humans to apply the test substance. This device can more colsely control the surface area exposed, the volume of sample in direct contact with the skin, the degree of occlusion, and the amount of sample leakage.

The adhesion chamber technique for testing skin irritation was first used by Rokstad (1940). In this method the test substance is placed in a circular depression chamber in the center of a celluloid plate. After positioning the patch at the site of application, the chamber is secured with adhesive tape while the skin is pressed up into the chamber.

Kurokawa et al. (1980) have reviewed the various types of patch test units and have divided them into several categories, including the following.

Table 1-9. Summary of Testing Procedures Used in Dermal Studies

Author	Method	Patch Type	Patch Size	Occlusion Method	Exposure Time	Observation Time	Scoring Evaluation	Misc.	Type Animal[a]
BENKE et al.	patch	Webril	.875 in dia.	yes; no method given	4 hr.	N.S.[b]	N.S.		MAN
BJORNBERG	Cup tests (Blohn, '60)				2 hrs.	24/72 hrs.	visual	controls	MAN
	Chamber tests (Rokstand '40)				24 hrs	24/48 hrs.	visual		MAN
	Open (Wedroff Dolgoff '35)				N.S.	immediate & 24/72 hrs.	visual		MAN
DAHL & TRANCIK	patch	Al test	N.S.	Blenderm tape	24 hr.	24,26,26,30 72,96 hours	visual		MAN
FINKELSTEIN et al.	patch	cotton flannel	1.25 in x 1.25 in	poly-ethylene & elastic bandage	5/17 hr. daily for 4-5 days	daily for 4-5 days	visual		MAN
FINKELSTEIN et al.	patch	cotton flannel	1.25 in x 1.25 in	poly-ethylene	varies with sub-stance tested	daily	visual		MAN
FROSH & KLIGMAN	Duhring chamber	Webril or Curity	12 mm dia.	none	24 hrs. than 6 hrs/day 4 days	36 hrs. after test finished	visual		MAN
FROSCH & KLIGMAN	chamber	Webril	12mm dia.	none	1x day for 3 day	72 hr.	visual		MAN
GRIFFITH & BUEHLER	patch	N.S.	N.S.	N.S.	4 hr.	4,24,48 hours	visual		MAN
JUSTICE et al.	arm im-mersion	none	none	none	3 x day 2 hr. in-terval 15 min. each	2 hrs.	visual		MAN
JUSTICE et al.	patch	gauze	N.S.	elasto-plast	18 to 24 hours	18 to 24 hours	visual		MAN

Table 1-9. Summary of Testing Procedures Used in Dermal Studies

(Continued)

Author	Method	Patch Type	Patch Size	Occlusion Method	Exposure Time	Observation Time	Scoring Evaluation	Misc.	Type Animal[a]
KLIGMAN	patch	nonwoven cotton Webril	1 in x 1 in	Blenderm	daily for 10 days	daily	visual		**MAN**
KLIGMAN & WOODING	patch	Webril	1 cm x 1 cm	Blenderm	until ery. or 10 days min.	each day	visual		**MAN**
KUROKAWA et al.	KI chamber	filter paper	N.S.	aluminum	24 hr.	1hr/24 hr.	visual		**MAN**
KUROKAWA et al.	Finn chamber	filter paper	N.S.	aluminum	24 hr	1hr/24hr.	visual		**MAN**
KUROKAWA et al.	Al test	filter paper	N.S.	aluminum foil & poly-ethylene	24 hr.	1hr/24hr.	visual		**MAN**
LANMAN et al.	patch	nonwoven cotton Webril	1 in x 1 in	Blenderm	daily until irritation observed	daily	visual		**MAN**
LARSEN	patch	N.S.	N.S.	aluminum backed	48 hrs.	48 or 72 hours	visual		**MAN**
MAIBACH & EPSTEIN	patch	N.S.	N.S.	occlusive dressing	7 to 12 days	every 24 hr.	visual	controls	**MAN**
MARZULLI & MAIBACH	patch	Webril	N.S.	blenderm	21 days cumulative	24 hr. than daily	visual		**MAN**
MOTOYOSHI et al.	patch	lint	15mm dia	none	48 hr.	48 hr.	visual		**MAN**
ODOM & MAIBACH	spread with glass rod	none	none	none	30 min	30 min	visual	contact uticaria testing	**MAN**
RAPPAPORT et al.	patch	N.S.	N.S.	N.S.	21 days continuous	daily or ev. 48 hrs.			**MAN**

Table 1-9. Summary of Testing Procedures Used in Dermal Studies

(Continued)

Author	Method	Patch Type	Patch Size	Occlusion Method	Exposure Time	Observation Time	Scoring Evaluation	Misc.	Type Animal^{a}
SHELANSKI & SHELANSKI	repeated insult	N.S.	N.S.	none	I. Ev 48 hrs. for 30 days; II.48 hrs.	ev. 48 hrs. phases 48 hrs.	visual	Two	MAN
SKOG	patch	N.S.	N.S.	N.S.	N.S.	24 hrs. after removal	visual		MAN
SKOG	rubbed for 2 min.	none	none	none	2 wks. 1w trtments	N.S.	gross micro.		MAN
SMEENK	patch	cotton lint	16 mm. dia	adhesive tape	24 hr.	24 hr.	visual		MAN
SMEENK	arm immersion	none	none	none	2x day for 30 min. for 5 days	N.S.	visual		MAN
SULZBERGER et al.	patch	gauze	3 in x 3 in	vaseline gauze elastic adhesive	24 hr.	24 hr.	visual		MAN
SULZBERGER et al.	patch	linen or cotton	1 cm x 1 cm	elasto-patch	24 hr.	24 hr.	visual		MAN
BENKE et al.	immer.	none	none	none	4 hr.	freq. intervals for 2 wks.	visual		GP
FINKELSTEIN et al.	patch	cotton flannel	1 in dia.	poly-ethylene	16 hr.	16 hr.	visual		GP
GRIFFITH & BUEHLER	patch	N.S.	N.S.	N.S.	4 hr.	4,24,48 hours	visual		GP
OPDYKE & BURNETT	immersion	none	none	none	4hrs. each for 3 days	72 hrs.	visual		GP

Table 1-9. Summary of Testing Procedures Used in Dermal Studies

(Continued)

Author	Method	Patch Type	Patch Size	Occlusion Method	Exposure Time	Observation Time	Scoring Evaluation	Misc.	Type Animal[a]
ROUDABUSH *et al*.	patch	cellulose	1 in x 1 in	"appropriate sleeve"	N.S. prob. DRAIZE	N.S. prob. DRAIZE	visual		GP
SKOG	rubbed for 1 min.	none	none	none	daily between 36 & 95 times	daily	visual histological		GP
HOMBERGER *et al*.	open	none	none	none	daily for 30 days	30 days	histological		HRL MICE
JUSTICE *et al*.	painting	none	none	rubber dam	every 10 min. for 70 min.	70 min.	histological		MUS
SCHMID	open	none	none	none	3 x wk for 8 wks	3 x wk	visual		MUS
SCHMIDT & EVANS	mouse ear	none	none	none	until irritancy	24 hr	visual		MUS
UTTLEY & VAN ABBE	mouse ear	none	none	none	daily for 4 days	daily	visual		MICE
BENKE *et al*.	patch	felt	0.75 x 1 in	elastoplast	24 hr.	24/72 hr.	visual		RBT
BROWN	patch	lint	2 cm x 2 cm	polyethylene	7 days	daily/7 days	visual	sulfan blue	RBT
BROWN	drip	none	none	none	4 1/2 wk. 5 day/wk	daily/5 days/week	and	trypan blue	RBT
DRAIZE	patch	gauze	1 in x 1 in	tape; rub.	24 hr.	24hr/72hr	visual	standard	RBT
FINKELSTEIN *et al*.	patch	cotton flannel	1 in. dia.	polyethylene	16 hr.	16 hr.	visual	formaldehyde & controls	RBT
GRIFFITH & BUEHLER	patch	N.S.	N.S.	N.S.	4 hr.	4,24,48 hours	visual		RBT

Table 1-9. Summary of Testing Procedures Used in Dermal Studies

(Continued)

Author	Method	Patch Type	Patch Size	Occlusion Method	Exposure Time	Observation Time	Scoring Evaluation	Misc.	Type Animal[a]
INGRAM & GRASSO	patch	Melolin	1.5 cm x 1.5 cm	Blenderm	5hr./day for 5 days	each day for 5 days	visual histological		RBT
MARZULLI MAIBACH	open	open	open	none	14 days cumulative	24 hr. each day for 15 days (16 readings)	visual & skin thick		RBT
MOTOYOSHI et al.	applied from syringe	none	none area 3 cm x 3 cm.	plastic collar	24 hr. & 2nd 24 hr.	24/48/72 hrs.	visual histological	Evans blue	RBT
STEINBERG et al.	patch	gauze	2 in x 2 in	elastic bandage	24 hrs.	24hr./48hr.	visual		RBT
	open	none	none	none	24 hrs.	24 hrs.	visual		RBT
	open	none	none	saran	24 hrs.	24 hrs.	visual		RBT
	patch	gauze	5 cm x 5 cm	saran	7 x wk	daily	visual		RBT
	patch	gauze	5 cm x 5 cm	elastic bandage	for 3 wks.	daily	visual		RBT
VINEGAR	patch	gauze	1 in x 1 in	rubber dam	24 hr	24hr./72hr.	visual		RBT
WOLVEN & LEVENSTEIN	patch	Webril	2.5 cm x 2.5 cm	Blenderm	24/4/1hr	24/4/1hr	visual	sulfan blue	RBT
MOTOYOSHI et al.	patch	N.S.	15mm dia.	rubber. cloth adhesive tape	48 hr.	48 hr.	visual histological		MINI SWINE
MOTOYOSHI et al.	applied from syringe	none	none area 3 cm x 3 cm.	plastic collar	24 hr. & 2nd 24 hr.	24/48/72hrs.	visual histological		GP RAT

Table 1-9. Summary of Testing Procedures Used in Dermal Studies

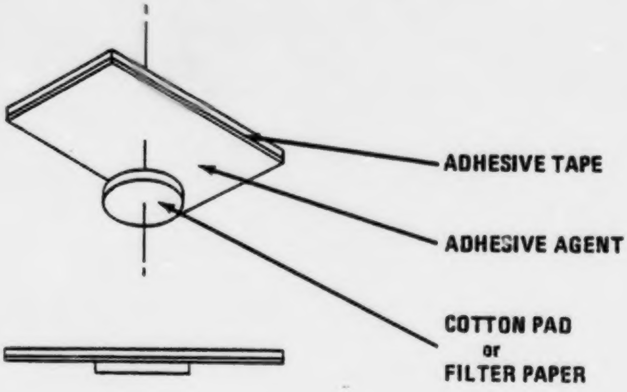
(Continued)

Author	Method	Patch Type	Patch Size	Occlusion Method	Exposure Time	Observation Time	Scoring Evaluation	Misc.	Type Animal[a]
DAVIES *et al.*	patch	gauze	1 in x 1 in	rubber cloth	48 hrs.	48,72 hrs.	visual		MICE, GP, DOG, RBT, PIG, BBOON, MAN

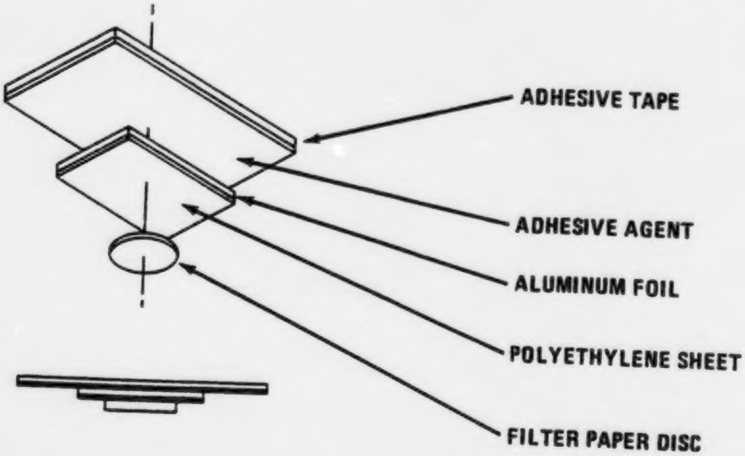
[a] GP - Guinea Pig
RBT - Rabbit
HRL - Hairless
BBOON - Baboon
MUS - Mouse

[b] Not Specified

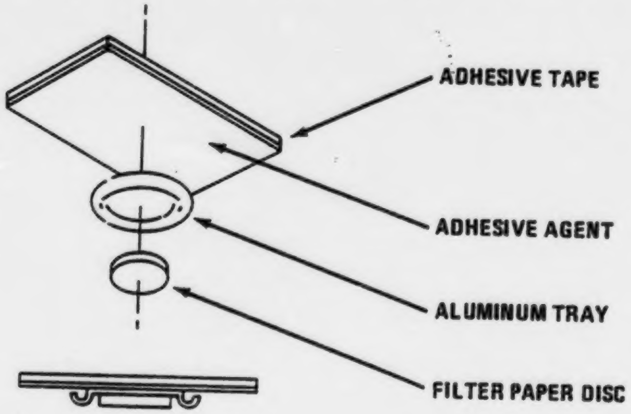
The standard cotton pad or gauze and adhesive tape has been the most widely used and studied. In this example, a cotton pad, gauze, or porous sheet is impregnated with or placed on the test substance and secured to the skin with adhesive tape. A potential problem with this technique is that the test substance may become contaminated with the adhesive material or leak onto surrounding areas. In addition, the tape is in contact with the skin proximate to the patch. These factors may influence the results of the test and thereby decrease reproducibility and accuracy.


ADHESIVE TAPE
ADHESIVE AGENT
COTTON PAD
or
FILTER PAPER

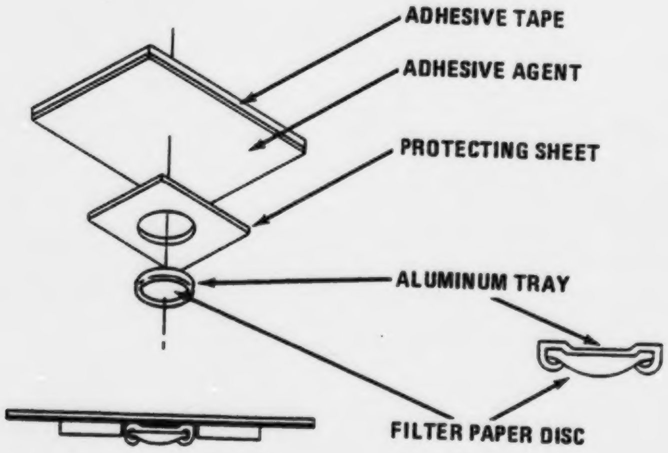
The Al-Test method of the International Contact Dermatitis Research Group uses a patch test unit that consists of polyethylene lined aluminum foil on to which a filter paper disc is placed. This procedure separates the area where the adhesive tape is placed from the test site and allows perspiration to flow into and out of the paper disc. Perspiration between the polyethylene sheet and the skin may influence the response.


ADHESIVE TAPE
ADHESIVE AGENT
ALUMINUM FOIL
POLYETHYLENE SHEET
FILTER PAPER DISC

The Finn Chamber, a tray, was designed to eliminate the contamination of the test substance by perspiration or the tape adhesive. The aluminum tray (no polyethylene is used) is shaped so that a filter paper disc fits inside which can be "sealed" to the skin by adhesive tape. Although this design greatly reduces the chance of contamination, the close proximity of the adhesive tape to the test area may alter the response.


ADHESIVE TAPE
ADHESIVE AGENT
ALUMINUM TRAY
FILTER PAPER DISC

The KI-Chamber is a device that was designed by Kurokawa and colleagues to eliminate the possible contamination of the test substance with perspiration or the adhesive. It consists of an aluminum tray which is inserted in a hole in a porous protective paper sheet. The filter paper disc is then placed in the tray. Thus the adhesive tape does not come into contact with the test area.


ADHESIVE TAPE
ADHESIVE AGENT
PROTECTING SHEET
ALUMINUM TRAY
FILTER PAPER DISC

Kurokawa et al (1980) compared the effectiveness of the KI chamber with the Al-test and Finn chambers in humans exposed to vehicles and irritants. They found that the KI chamber showed fewer false positive reactions with vehicles and was superior to the other patch test units in detecting water soluble irritants.

Frosch and Kligman (1979) have also designed a patch test unit, the Duhring Chamber, which is similar to the Finn chamber but has a larger inner diameter. An elevated flange indents the skin in a circular pattern thus preventing leakage of the test substance. A comparison of the two chambers showed that the Duhring chamber produced reactions that were uniformly more intense. The authors conclude that this increased sensitivity was due to the fact that twice as much test agent can be applied to a given area of skin with the Duhring chamber. On removing the chamber, an imprint or pressure ring on the skin indicates that total occlusion was used during the test. Another advantage is that the area of skin tested and the amount of test agent can be standardized.

4.1.1 Immersion

Immersion has been used in attempts to reduce the variability of test results and increase the sensitivity of the test subjects. Benke et al. (1977), Opdyke and Burnett (1965) and MacMillan et al. (1975) used an immersion technique to evaluate the irritancy potential of aqueous solutions in the guinea pig. Good agreement was found between this technique and the Rabbit 5-Day Dermal Irritation (Patch) Test (MacMillan et al., 1975). The technique of immersion has not been validated in the rabbit.

"Human Arm Immersion" has been used to increase sensitivity to mild irritants in humans (Polano, 1968; Smeenk, 1969; and Justice et al., 1961). This technique, as with repeated patch tests, is based on the principle of elicitation of response by repeated exposures of the same area to the test material. The number of exposures required to achieve a prescribed level of irritation in this sense amounts to a quantal response. This technique is generally suitable for water soluble substances (soaps). The arm immersion test method allows the standardization of the exposure conditions. Statistical evaluation of the arm immersion technique has shown that it is reproducible (Justice et al., 1961). Disadvantages are that it could induce systemic toxicity if the test material were readily absorbed and it requires a large amount of test material.

4.1.2 Occlusion

Steinberg et al. (1975), and Phillips et al. (1972) tested a variety of compounds under different occlusive conditions (e.g., saran and stockinette, elastic bandage, and both elastic bandage and stockinette). The type of occlusion had a significant effect on the irritation response of the rabbit to the test compound, thereby producing a change in its numerical score and the irritation category.

Gilman et al. (1978) demonstrated the effect of occlusions on dry and moistened samples of commercial detergents in the rabbit. Occlusive patches gave 1.5 to 10 times higher Primary Irritation Indices when compared to semi-occlusive patches. The longer the patch was in place, the more severe the response and the more marked the difference between the two patches. The results are shown in the following table.

Primary Dermal Irritation With a Detergent in Rabbits

	Dry Powder		Paste (0.5 g + 0.1 ml)	
Exposure Time (Hours)	Occluded	Semi-occluded	Occluded	Semi-occluded
4	0.2	0.0	0.0	0.0
8	0.4	0.2	0.3	0.05
16	1.3	0.5	2.0	0.25
24	3.4	0.3	7.1	1.3

The use of occlusive patches possibly puts the skin under duress, impedes the normal functions of sweat evaporation, cooling, and respiration, and interferes with evaporation of the test compound. As a result, skin permeability and sensitivity are increased, which often leads to a "false positive" reaction (Idson, 1969; Lanman et al., 1968; Rostenberg, 1961). This was further illustrated by Phillips et al. (1972) who examined the occlusive and nonocclusive (open) conditions of testing. A majority of the compounds that caused some reaction under occlusive patch testing caused no reaction under open conditions. Magnusson and Hersle (1966) studied occlusion to detect subtle irritation. The use of the occlusive patch statistically decreased the incidence of false negative responses with water-based irritants and petrolatum bases by increasing the degree of irritation when compared to non-occluded sites.

Similiar observations suggest that adequate assessment of primary irritants may require both open and occlusive tests if very high sensitivity is required for special use and applications (Battista and Rieger, 1971; Skog, 1963; Rostenberg, 1961).

4.1.3 Conclusions on Patch Test Unit

In summary, the use of chambers to hold the test material in contact with the skin in dermal irritation studies is a promising technique that deserves further evaluation in animals. A number of investigators have concluded that the chamber increases reproducibility. Since total occlusion produces a substantial increase in sensitivity, substances that are nonirritating by other methods may be weakly irritating when tested with a chamber. Another advantage of the use of chambers is that the amount of chemical and the test area can be standardized. Leakage from the patch site is also decreased. The use of chambers may, however, increase costs in the short run. Immersion is another promising human procedure which should be given further consideration for use in animals.

4.2 Abrasion

The standard Primary Irritation Index (PII) (Table 1-3) is derived from scores equally weighted from intact and abraded skin. Abraded skin generally gives a more severe reaction than intact skin because destruction of the stratum corneum removes the barrier for penetration. The concentration of the test chemical needed to produce an effect is, therefore, usually lower. Standardization of abrasion techniques is also believed to be difficult. For these reasons, the National Academy of Sciences (1977) recommended against using abraded skin, citing the data of Nixon et al. (1975). These data showed that rank order classifications of irritation based only on intact skin usually resembled those for abraded skin. Nixon and his coworkers (1975) stated that most test materials gave irritation scores in animals, especially rabbits, that were considerably higher than human scores. They attributed this discrepancy to the much higher scores obtained from abraded skin. This is shown clearly in Table 1-10. These authors came to the same conclusion as the National Academy of Sciences on the testing of abraded skin. They explained that the Draize test was originally designed to test topical drugs and cosmetics where damaged skin may be present.

McCreesh and Steinberg (1977) found no differences in irritation when 5 materials were tested in rabbits on intact skin and skin abraded by 3 different techniques. They also cited the finding of Nixon et al. (1975) that no correlation existed between scores on abraded skin of rabbits and the potential for a compound to be a primary skin irritant in humans. Therefore, if there are no differences between abraded and intact skin, or if abrasion gives an exaggerated response, abraded skin testing is an unnecessary step as test results will be misleading.

A scarification (abrasion) technique was used by Frosch and Kligman (1977) in human subjects to assess the irritancy of a large number of agents. Having first experimented with scotch tape stripping, abrasion with hard particles, pretreatment with anionic surfactants and the use of dimethylsulfoxide (DMSO) to alter the stratum corneum, they were unable to demonstrate any consistent reproducibility. The most satisfactory technique consisted of scratching with a 30-gauge half-inch needle. Using the Duhring chamber (Frosch and Kligman, 1979) sealed to the skin by a nonocclusive tape, the test agent was applied once daily for 3 days. Daily readings were taken and reactions graded from 0 to 4. The ratio of the mean irritancy thresholds on scarified and normal skin was then determined and designated as the scarification index (SI). Substances believed to be nonirritating produced no reaction, but most irritants were detectable at much lower concentrations using scarification, indicating that abrasion increases the sensitivity of the test. The authors suggested that the increase in sensitivity and reproducibility should justify the use of the scarification technique.

Frosch and Kligman (1977) reported that scarification (abrasion) greatly increased sensitivity and reproducibility for the detection of weak irritants in humans. They measured the lowest concentration at which the test substance caused irritation. This was identified as the threshold concentration. They recognized that abrasion may be inappropriate for strongly irritating substances but emphasized that a high threshold concentration (low score) with

Table 1-10. Four-Hour Patch Test Results[a]

Test material	Concentration (w/v aqueous)	Animal species	Mean Scores[b]		Irritancy Judgement[c]
			Intact	Abraded	
Metasilicate/carbonate detergent granules	50%	Rabbit	6.8	8.0	Corrosive
		Guinea pig	0.0	0.6	Negligible
		Human	3.0	4.2	Severe
High carbonate detergent granules	50%	Rabbit	0.9	2.6	Negligible
		Guinea pig	0.0	0.4	Slight
		Human	0.0	0.0	Negligible
Low carbonate detergent granules	50%	Rabbit	0.7	0.8	Slight
		Guinea pig	0.1	1.0	Slight
		Human	0.0	0.2	Negligible
Phosphate detergent granules	50%	Rabbit	1.2	5.6	Moderate
		Guinea pig	0.2	1.0	Slight
		Human	0.0	0.4	Negligible
Enzyme detergent granules	50%	Rabbit	1.3	4.3	Moderate
		Guinea pig	0.0	0.6	Negligible
		Human	0.0	0.1	Negligible
Liquid detergent	undiluted	Rabbit	2.4	3.1	Moderate
		Guinea pig	0.8	1.6	Slight
		Human	0.1	0.1	Negligible
Liquid cleaner	undiluted	Rabbit	3.0	5.2	Moderate
		Guinea pig	0.3	4.3	Moderate
		Human	0.2	0.5	Negligible
Pine oil cleaner	undiluted	Rabbit	3.6	4.0	Moderate
		Guinea pig	2.5	3.1	Moderate
		Human	1.0	0.6	Slight
Household ammonia	undiluted	Rabbit	1.4	4.0	Moderate
		Guinea pig	0.0	4.0	Moderate
		Human	1.5	---	Moderate
Hypochlorite bleach	5.25%	Rabbit	1.0	1.3	Slight
		Guinea pig	0.3	1.2	Slight
		Human	3.9	---	Severe
Liquid shampoo	undiluted	Rabbit	3.7	4.2	Moderate
		Guinea pig	2.1	2.3	Moderate
		Human	0.9	1.4	Slight

Table 1-10. Four-Hour Patch Test Results[a]

(Continued)

Test material	Concentration (w/v aqueous)	Animal species	Mean Scores[b]		Irritancy Judgement[c]
			Intact	Abraded	
Antiperspirant	undiluted	Rabbit	0.0	1.0	Slight
		Guinea pig	0.0	0.0	Negligible
		Human	0.1	0.5	Negligible
Coconut soap	50%	Rabbit	2.5	3.1	Moderate
		Guinea pig	0.3	0.8	Slight
		Human	0.0	0.0	Negligible
Isopropyl alcohol	undiluted	Rabbit	0.0	0.0	Negligible
		Guinea pig	0.0	0.0	Negligible
		Human	0.0	0.8	Negligible
Lemon juice	undiluted	Rabbit	0.0	1.0	Slight
		Guinea pig	0.0	0.0	Negligible
		Human	0.2	0.3	Negligible
Table salt	50%	Rabbit	0.0	0.5	Negligible
		Guinea pig	0.0	0.0	Negligible
		Human	0.0	0.8	Negligible
Potassium hydroxide	10%	Rabbit	6.9	7.0	Corrosive
		Guinea pig	7.6	7.6	Corrosive
		Human	---	---	Corrosive
Sodium metasilicate	50%	Rabbit	8.0	8.0	Corrosive
		Guinea pig	1.7	3.2	Moderate
		Human	---	---	Corrosive
Sodium carbonate	50%	Rabbit	0.0	1.5	Slight
		Guinea pig	0.0	0.1	Negligible
		Human	0.0	2.0	Negligible
Sodium tripolyphosphate	50%	Rabbit	0.0	0.5	Negligible
		Guinea pig	0.0	0.0	Negligible
		Human	0.0	1.8	Negligible
Sulfuric acid	10%	Rabbit	0.0	0.1	Negligible
		Guinea pig	0.0	0.0	Negligible
		Human	0.2	0.2	Negligible
Acetic acid	10%	Rabbit	0.0	1.7	Slight
		Guinea pig	0.0	0.1	Negligible
		Human	1.0	1.1	Slight

Table 1-10. Four-Hour Patch Test Results[a]

(Continued)

Test material	Concentration (w/v aqueous)	Animal species	Mean Scores[b] Intact	Abraded	Irritancy Judgement[c]
Sodium lauryl sulfate	50%	Rabbit	1.6	2.6	Moderate
		Guinea pig	0.0	0.8	Negligible
		Human	0.6	1.1	Slight
C_8C_{10} fatty acids	undiluted	Rabbit	4.4	4.7	Moderate
		Guinea pig	0.1	0.8	Slight
		Human	2.3	---	Moderate

---

[a] Adapted from Nixon et al. (1975).

[b] Sum of mean erythema and edema scores (on a 0-4 scale) at 4, 24, and 48 hr.

[c] Irritancy judgements for the human scores are according to the following scale (for intact skin sites only): 0-0.4 = negligible; 0.5-1.4 = slight; 1.5-2.4 = moderate; 2.4 = severe; substantial tissue destruction or irreversible change = corrosive. For both animal species, the scale is based on PII scores, as follows: 0-0.4 = negligible; 0.5-1.9 = slight; 2.0-4.9 = moderate; 5.0-8.0 = severe; tissue destruction or irreversible change = corrosion (Draize, 1959).

abrasion is a guarantee of mildness no matter how the product is used. The main objective of their work was to develop methods to detect mildly irritating compounds and/or those causing irritation in a small fraction of the population. Organic acids, antimicrobials, inorganic salts, and surfactants were tested with and without abrasion. In every instance, irritation was detected at a lower concentration on abraded skin (2.5- to over 100-times lower) than on intact skin (Table 1-11).

In summary, the value of abrasion in dermal irritation testing depends on the exact purpose of the test. Abrasion could be used to increase sensitivity in the testing of skin preparations or detergents where irritation in a small percentage of the population is to be avoided whenever possible. When measuring the corrosive potential of a chemical for a DOT shipping label where the extra sensitivity is not required, abrasion would be seen as causing an exaggerated response.

An initial test on intact skin should be performed. If no irritation is noted and increased senstivity is required, the test could then be performed on abraded skin. If a low score is again obtained, the mildness of a product is further assured. The added sensitivity of the abraded skin test should continue to be optional in the general case for industrial chemicals but may be useful in more fully assessing irritation potential.

4.3 Application of Dry Test Substances

For animal experiments, most methods utilize 0.5 g or 0.5 ml on an inch square area, as was originally recommended by Draize. Although the exact method of application of solids and semi-solids is not clearly defined, the sample usually is moistened before it is applied. Liquids are generally applied undiluted but a dilute test substance may be justified based on the use of the material in its final form. For solids or semi-solids, considerable variation in technique is possible within the framework of the proposed guidelines. For example, a solid may be applied dry to premoistened skin, dry and covered with a moistened patch, dry and "injected" beneath a preapplied wet patch, dry or as a slurry. Sullivan et al. (1975) examined these variables and found signficant differences in irritation. Depending on whether the material tested was applied dry and subsequently moistened or applied as a slurry, response ranged from no response to necrosis. McCreesh and Steinberg (1977), however, reported that the different methods of application had no effects on irritant ranking (although there were some differences in scoring and classification). They cited the findings of Steinberg et al. (1975) in which an evaluation of 12 compounds showed similar irritancy when the results for application to rabbit skin on a fixed area (2x2 cm) were compared with those for direct application under or on a gauze pad. They concluded that the physicochemical nature of the test agent is more important than the method of application.

Gilman et al. (1978) tested a number of detergents as a dry powder (100%), aqueous paste (83%) and as aqueous suspensions (50, 25, and 10% w/v) in rabbits. The degree of wetness was found to influence the degree of skin irritation. The most severe skin reactions occurred when the detergent was applied as a paste for 24 hours with an occluded patch.

Table 1-11. Effect of Abrasion on Skin Irritation in Humans[a]

Agent	Solvent	Threshold Concentration (%) Normal Skin	Threshold Concentration (%) Abraded Skin	Normal/ Abraded
Acids				
Oleic acid	ethanol	30.0	5.0	6
Benzoic acid	ethanol	30.0	7.5	4
Lauric acid	ethanol	4.0	1.0	4
Linoleic acid	ethanol	20.0	5.0	4
Antimicrobials				
Formalin	water	2.0	0.05	40
Triclosan	ethanol	1.5	0.25	6
Benzalkonium chloride	water	0.2	0.05	4
Hexachlorophene	ethanol	2.5	1.0	2.5
Inorganic Salts				
Nickel sulfate	water	20.0	0.13	154
Aluminum chloride	water	30.0	2.5	12
Potassium iodide	water	60.0	5.0	12
Surfactants				
Isostearamidopropyl morpholine lactate	water	25.0	2.5	10
Stearamidopropyl dimethylamine lactate	water	10.0	0.5	20
Triethanolamine	ethanol	100.0	5.0	20
Triton X-100	water	50.0	1.0	50
Sodium lauryl sulfate	water	0.5	0.05	10

[a] Adapted from Frosch and Kligman (1977)

The OECD guidelines specify that solid test substances should be moistened sufficiently with water or, where necessary, a suitable vehicle, to ensure good contact with the skin. Similarly, other methods call for premoistening solid test materials with water or an appropriate solvent.

Water probably should be the standard choice for moistening the test agent. One reason for using water is to mimic the presence of perspiration. To standardize the procedure for testing solids it would be advantageous to choose one condition such as a slurry with water applied to the patch at a 1:1 ratio (w/v) or any other convenient concentration. Another alternative would be to apply a dry powder and cover with patch soaked with 0.5 ml water. This would alleviate some of the imprecision in applying solid substances. Standardization of the method for moistening solid test substances would increase the ability of the test to assess the comparative irritancy of these materials. Extra tests with other solvents could also be performed to simulate conditions of use or exposure.

4.4 Application Site

The region of the body where the patch is applied can affect the results of irritation studies. In humans, patches are usually placed on the arm or on the back, while the abdomen and back are used most often in animals. The Federal Hazardous Substance Act does not specify the region on the rabbit to which the test substance should be applied, although a revision proposed in 1972 (by FDA) specified the back as the test site (FR No. 27, 27635-27636).

Vinegar (1979) examined the regional variation of primary skin irritation in rabbits. Thirty three common types of household items were tested with a modified Draize procedure. The Primary Irritation Indices of the abdomen were significantly higher than those of the back. A possible explanation of different skin thickness was offered. This study clearly demonstrated that the site of application is a potential source of variation between laboratories in skin irritation studies. Studies on the regional variation in skin irritation in humans were not identified in a search of the literature. However, Maibach et al. (1971) found marked regional variation in the percutaneous absorption of pesticides in man. Because the degree of irritation may be related to the rate of percutaneous absorption, regional variation in skin irritation probably also occurs in humans.

In order to minimize inter-laboratory variation a single skin site for testing should be specified. In animals, the back has been used most frequently. This is advantageous since access by the animal to the patch is minimized preventing resultant interference with the test site as well as ingestion or inhalation of the test substance.

4.5 Exposure And Observation Period

The original Draize test and FHSA guidelines (Table 1-3) specified a 24-hour exposure. The NAS recommended a 4-hour exposure based on a more realistic simulation of the type of exposure expected in man. Similarly, the IRLG and OECD guidelines specify a 4-hour exposure period. This does not preclude the use of longer exposures when warranted. When very large numbers of people might be exposed or when extended exposure is possible under anticipated conditions of use, a longer exposure period may be desired. The original Draize test, being intended for the study of cosmetic and topical agents, was designed to detect mild irritation that might occur in a very small proportion of exposed people. A substance that is not irritating after a 4-hour exposure may be irritating after a 24-hour exposure. This was shown by Gilman et al. (1978) for four dry detergent powders.

Primary Dermal Irritation Indices with Detergents in Rabbits

	PII	(See Table 1-2)
Sample	4-hr Exposure	24-hr Exposure
A	0.0	--
B	0.0	4.7
C	0.0	3.9
D	0.0	3.4

The authors considered these 24-hour irritation indices to be exaggerated, because even with misuse, exposures to detergents would be expected to be less than 4 hours. An increase in sensitivity with prolonged exposure was also demonstrated in humans by Kligman and Wooding (1967). Therefore, as is found for abrasion, the use of an extended exposure period is an effective means of achieving heightened sensitivity, when required.

While the current method for measuring skin irritation is usually satisfactory for detecting severe irritants, it lacks the ability to differentiate between a mild and a moderate human irritant (Phillips et al., 1972; Roudabush et al., 1965; Steinberg et al., 1975, NAS, 1977), and may not even rate them as irritating. Selectively increasing the exposure period in both animal and human studies may help in the determination of comparative irritancy. To obtain responses to mild and moderate irritants, extended periods of exposure have been suggested (e.g., 21-day continuous closed patch test). With this procedure in humans, the test material is applied to subjects under an occlusive patch for 24 hours before the patch is removed for evaluation. After scoring, the test material and patch are reapplied daily to the same site for 21 days (Steinberg et al., 1975). When this procedure was used, the authors found that sensitivity increased to a point where it agreed with data for rabbits treated under a similar regimen. This protocol is an adaptation of the repeated patch test for humans (Kligman, 1961), whereby the test material and patch are reapplied for a maximum of 10 days or until inflammation appears.

A major benefit of the 21-day continuous closed patch test is that it considerably reduces errors arising from subjective assessment of skin reactions scored after a single exposure of 24 hours, and thus offers better predictability of the irritant effects of test chemicals in man (Lanman et al., 1968). The added sensitivity gained, however, is not without its drawbacks and the 21-day test has been termed "economically heroic." (Steinberg et al., 1975).

In the Rabbit 5-Day Dermal Irritation Test devised by MacMillan et al. (1975), the test material is washed off after 4 hours of contact and the irritation is scored 20 hours later, according to the Draize method. Application of the material to the same site and scoring of the irritation are performed on 5 consecutive days. After removing the test substance, a period of 30-60 min. is allowed to elapse before scoring the site to minimize pressure and hydration effects. Readings are also usually taken at 48 and 72 hours after the patch was originally applied. The observation period is generally 7 days because corrosive effects are best seen at this time (NAS, 1977). It may be advisable to observe the animals for 2 weeks but practical considerations such as cost and usefulness of information should be taken into account.

In conclusion, four hours appears to be an adequate minimum exposure time for measurement of primary irritation from exposure to industrial chemicals. This time interval is specified by the IRLG and OECD guidelines and is compatible with DOT requirements. Many investigators may still choose to continue exposure for 24 hours or longer when increased sensitivity is required. The extent of both the exposure and observation periods remains primarily a matter of individual preference and need.

4.6 Enhancements to Visual Scoring

When the skin reaction to the standard patch test for skin irritants (especially with mild irritants) is difficult to detect (because the erythema is masked by the skin color of the animal), vital dyes or formaldehyde have been applied to increase sensitivity and to enhance the visibility of the erythemal response. Evans, Sulfan or Trypan blue are injected intravenously in animals being patch tested; the dyes collect in areas where vasodilation has occurred, coloring the skin within minutes after injection. This technique has proven useful in identifying mild erythema which was undetectable by the naked eye (Brown, 1971; Finkelstein et al., 1963; Wolven and Levenstein, 1967; Motoyoshi et al., 1979).

5.0 REPRODUCIBILITY AND SCORING

5.1 Reproducibility

Weil and Scala (1971) examined the reliability and reproducibility of skin irritation testing. Even though many toxicology laboratories attained a degree of internal consistency using their own unique experimental designs, no information had existed on the consistency of results among laboratories. Neither the results using the methods specified in the FHSA nor individualized laboratory methods had been compared. In a similar study in 1967, Weil and Wright examined the question of interlaboratory variability in acute oral toxicity testing. The assumption was that an oral LD50 determination required fixed methodology in order to attain reliability and reproducibility. The authors concluded that this was not the case but rather, competent laboratories will generally be in good agreement even though methods vary.

A different conclusion was reached by Weil and Scala (1971) for dermal irritation testing. In their study, a total of fourteen materials were selected and aliquots of single lots of each were coded and sent to over 20 participating laboratories for blind testing generally using FHSA testing methodologies. The results showed considerable variation between laboratories and at the same laboratory among rabbits treated with the same substance. The variability in scoring was marked in several areas including the range of scores for the same substances, the range of animals with necrosis, the presence or absence of recovery during the 72 hour grading period for the same substance, and the rank ordering of the substances for relative irritancy. One laboratory had edema and erythema scores of zero for every rabbit for each of 9 materials. The other laboratories scored these same materials much higher, in some cases, near the maximum possible score (23) for primary irritation.

Unlike FHSA (Table 1-3), the reference scoring system used by Weil and Scala scored necrosis as well as erythema and edema in determining the primary irritation score. Necrosis was scored as zero (no necrosis) for several compounds at one group of laboratories while the same compounds caused necrosis in a large percentage of the rabbits at several other laboratories. Recovery (decreased irritation) was apparent at 72 hours in some laboratories, while others reported much more severe reactions at 72 than at 24 hours. There was some sharp disagreement between labs on the relative ranking of several substances. When the average of all laboratories' scoring was compared with individual laboratory results, however, reference results correlated well in 20 of 22 laboratories and nonreference methods in 17 of 20, (in the other laboratories, there was no correlation.) The average responses when rank ordered by degree of irritation also agreed fairly well between reference and nonreference methods. The greatest variation occurred among the lowest irritation scores. This good correlation was not unexpected as the nonreference methods were in most cases either the FHSA or Draize procedures.

A follow-up study carried out at two laboratories, revealed that one rated compounds more severely than the other. This was considered by Weil and Scala to be a possible explanation for many of the variations in scoring. A steering committee member also performed the tests in each laboratory for comparison. There were apparent and very obvious differences in the application and wrapping procedures used. In both laboratories, readings were more severe when the laboratory personnel applied the test substances in comparison with the steering committee member. In one laboratory, the plastic wrapping was applied tightly and completely occluded the patch, therefore enhancing irritancy. Scoring in both laboratories came closer to the average scores for all laboratories in the test program when rerun, but they were still different from the "standard" results of the steering committee member. In one laboratory, the severe scores were due to the use of a different technician the first time. The steering committee member also obtained different readings in the two laboratories.

Weil and Scala (1971) concluded that although the Draize procedure had been in use in the various test laboratories for over 20 years, several laboratories were outliers. Since the type of laboratory; i.e. consultant, government, food, cosmetic, or industrial, had no relationship to its internal or external variability, they felt that the Draize test should not be used to classify materials as irritants when consistency was desired. Subjective scoring seemed to be the main source of error with procedural variations also contributing. As a solution to the problem the authors advised that training courses and clinics be conducted frequently.

5.2 Other Scoring Methods

Inspection of Tables 1-1, 1-3, 1-8, and 1-9 show that nearly all studies have utilized visual scoring. To minimize subjectivity and improve objectivity of the standard test results, several investigators have used additional quantitative techniques to identify skin damage caused during testing more clearly. Ingram and Grasso (1975) found a good correlation between the histologic and visual evaluations of irritant induced changes. Histological examination was used by Guillot and coworkers (1981) to evaluate irritation responses where the color of the test agent (e.g., copper nitrate, orthovanillin) precluded visual scoring. A scoring system based on histopathologic examination was also used by Brown (1971). Other approaches, such as measurement of dermal respiration and enzymatic activity, alterations in dermal collagens, changes in dermal pH, elasticity, and electrical properties, have been discussed by Lansdown (1972).

A simpler quantitative procedure, which is much less dependent on expensive equipment or limited resources (pathology), was originally described by Kligman and Wooding (1967) in humans. The sole criterion of irritation in this method is the presence or absence of erythema. The data are subjected to a statistical analysis, similar to that used in determining an LD50, and the values obtained can be used to assess the relative irritancy of materials.

Test agents are applied to a Webril patch which is occluded to the skin by overlapping strips of impermeable plastic tape. A 1 square centimeter patch is used based on the results of quantitative measurements which showed that above 0.5 cm2, the size of the patch does not affect results. Mixtures containing volatile substances (i.e., alcohol, acetone) at concentrations of 20% or more are tested with a nonocclusive patch since the strong irritation produced by the volatile components masks the effects of any other ingredients. In these tests, Webril patches (1 cm2) are held on the skin under slightly larger squares of gauze and are fastened only along the edges with perforated tape. Test materials are applied in a volume which loads the patch completely without producing overrun (approximately 0.05 ml/cm2).

For weak irritants, an IT50 value (the number of days required to cause 50% of the sampled population to develop a threshold irritant response) is obtained per each irritant tested in a minimum of 10 subjects exposed continuously for 10 days. Patches and irritants are applied once daily at the same site. An end point is reached when positive erythema is noted. The number of days required for this response is recorded. The logarithm of the cumulative percentage of animals showing a reaction is plotted against days and the IT50 is obtained from the resulting line.

For tests of strong irritants, exposure lasts only one day. Different concentrations are used and the logarithm of the cumulative percentage of animals showing a reaction is plotted against concentration. In this case, an ID50 value (the concentration which produces an irritant response in 50% of the sampled population) is determined.

IT50 and ID50 values represent relative assessments of irritancy which are most meaningful when a standard of reference is included in the test. A similar approach was used by Justice et al. in 1961.

Steinberg et al. (1975) have suggested the use of a Threshold Irritation Concentration (TIC) of test chemicals in this type of testing. This resulted from their observation that a good correlation exists between the threshold concentration and the corresponding irritation score. Nevertheless, the results of repeated patch testing could be misleading in distinguishing between products that cause primary irritation and the general effects of repeated patching, sometimes termed "skin fatigue".

5.3 Recommendations

Standardization of scoring procedures combined with improved training guidelines can minimize variation in subjective scoring. FDA's Proposed Revision of the test for Primary Skin Irritants (37 CFR 244, p.27635) described the first step in such training efforts as an "Illustrated Guide for Grading Dermal Irritation." Thus far, only a guide for eye irritation has been published (formerly available from the Consumer Product Safety Commission). It is strongly recommended that a series of photographs or slides depicting various grades of irritation be produced. The reproductions could be evaluated and "graded" by a suitable panel of toxicologists. Ultimately a guide, much the same as that originally developed by the FDA for eye irritation, could be made available for public dissemination. This would greatly facilitate the training of personnel in dermal irritation studies and could help standardize the scoring. Photographs of the various degrees of occlusion would also be valuable. The validation of the quantal response method in animals described by Kligman and Wooding (1967) should also be pursued as this less subjective method would markedly reduce the sources of error outlined by Weil and Scala (1971). Solicitation of specific information on the parameters used in dermal irritation studies by laboratories is also suggested.

6.0 CONCLUSIONS

The rabbit is the species most widely used for dermal irritation testing although the guinea pig shows similar sensitivity and is more economical to house. Both species do not completely predict human response, however, as moderately and minimally irritating compounds may show either stronger or weaker response. In many cases, the rabbit and guinea pig results taken together more accurately predict the human response; compounds misgraded by one species are more accurately detected by the other. The final measurement of human potential exposure must be tested in man. The hairless mouse has received attention recently as an alternative test species. Both the guinea pig and the hairless mouse seem to deserve further study.

The use of chambers is becoming more common in human testing. These devices show several advantages over the standard guaze patch. Leakage from the test site is decreased and the degree of occlusion, amount of chemical and area of exposure are more effectively controlled when chambers are used. No one chamber design seems to be superior to the others at this time. The chamber has not been validated in animals; this action is strongly recommended. Immersion has also been used successfully in humans and appears promising but needs to be examined further in animals.

Occlusion of a patch test site affects the sensitivity of the skin to irritants as does abrasion. There is a difference in viewpoint whether this is an increase in sensitivity or an exaggerated response depending on how the results of the test are used. Neither full occlusion nor abrasion is recommended for general testing but should be allowable modifications if more sensitivity is required by the testing organization.

Solid materials are less easily handled than liquids and the method of application can affect the degree of irritaion. Water is the most common solvent used for moistening solids.

The site of application can also affect results, the abdominal region being more sensitive than the back. The dorsal region is preferred as it is easily accessible to the animal handler but not to the animal.

Four hours appears to be an adequate minimum exposure time for the large majority of compounds that would require testing. Increasing the time of exposure to a compound will increase skin sensitivity. Investigators have used periods up to 21 days to enhance effects of very mild irritants.

Scoring, as it is currently practiced, is a rather subjective procedure. At present, there are no standard study sets available for training as there are for eye testing. Several approaches to measure quantal responses have been described for human testing but need further evaluation in animals.

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DERMAL SENSITIZATION

1.0 SUMMARY

Dermal sensitization or contact dermatitis is a delayed allergic response to a substance applied to the skin in which the clinical manifestations are similar to those observed in dermal irritation, i.e., erythema and edema.

The guinea pig is the animal model used most frequently for contact dermatitis testing. The most common procedures employed for determining dermal senzitization in the guinea pig can be divided into three groups according to the method of administration of the potential allergen.

- Intradermal injection - used in the Draize and the Freund Complete Adjuvant Tests.
- Topical application - used in the Open Epicutaneous Test and the Closed Patch Test.
- A combination of intradermal injection and topical application - used in the Maximization, Optimization, Split-Adjuvant and the Footpad Tests.

These test procedures are reviewed and evaluated for their efficacy as predictive tests.

Comparative data from various sources indicate that all the test procedures except that of Draize have some potential for detecting "weak" sensitizers. The identification of weak sensitizers is extremely important in the testing of cosmetics and toiletries. However, no one test is suitable for testing all compounds and wide variation in the data from different sources using the same test procedure, compound, and concentrations indicates the need for a more thorough and systematic evaluation and validation of the test protocols. Test procedures requiring intradermal injections are inappropriate for use on finished products such as those containing emulsifiers, bacteriostatic agents and formaldehyde.

The Maximization test is one of the better validated tests for determining weak sensitizers but the Optimization, Open Epicutaneous and the Closed Patch tests appear to be viable alternatives. More published evidence, however, is needed to confirm the reliability of the latter three test methods.

In humans, the Maximization test procedure was found to be more sensitive than Schwartz-Peck, Shelanski-Shelanski and the Draize tests, especially for detecting "weak" allergenic reponses. Some problem with variation in results exists due to the different modifications of the Maximization test that have been used for human testing. Therefore, this procedure needs to be studied further. The results of the Maximization test indicate that substances which sensitize guinea pigs also sensitize humans. The quantitative agreement between the human and guinea pig response to a variety of test chemicals is also good.

A practical skin sensitization testing program for a wide variety of materials, such as industrial chemicals, pesticides, new drugs, paints and coatings, toiletries and cosmetics should use both the guinea pig and man as test subjects. Positive (strong sensitization) results in the guinea pig would suggest the unsuitability of the substance where use exposure is likely. A weak or negative response would indicate the need for further characterization and testing in small groups of human subjects using the Maximization test.

2.0 INTRODUCTION

2.1 Objective

The objective of this survey is to characterize, compare, and evaluate the eight most common dermal sensitization testing procedures employed in the guinea pig with the goal of determining which of the test methods is most predictive of the human sensitization response. At present these eight methods are approved for use by the OECD (1981) guidelines for measuring dermal sensitization.

The eight procedures described are the Draize, Freund's Complete Adjuvant, Open Epicutaneous, Closed Patch, Maximization, Optimization, Split-Adjuvant and Footpad Tests.

2.2 Scope

This report evaluates tests for identifying potential allergens. In some instances, however, compounds appear to act more subtly and require repetitive contact or special environmental conditions (increased temperature and humidity, occlusion, provocation, etc) to produce a response. Thus, no single test or group of test will correctly identify all potential allergens under all circumstances.

Scoring and grading charts are included when necessary to illustrate differences in criteria and test results and the correlation among findings of individual investigators or test laboratories. Data from different procedures are used to compare the efficacy (or lack of it) of the procedures for detecting grades of sensitization; e.g., strong, moderate or weak allergens.

Test procedures used in human sensitization are reviewed briefly. The review is limited to those procedures for which comparable data were found in the guinea pig. The comparative data were used to show the similarities and differences in allergenic responses between the human and the guinea pig under similar experimental conditions and to illustrate the predictability and reproducibility of the human response from animal experiments.

The comparative data from a diagnostic test on humans from different geographic areas (the North American Contact Dermatitis Group and the International Contact Dermatitis Research Group) were included to illustrate the limitations involved in extrapolating data from test animals to humans or results from small localized test populations under closely defined environmental conditions to the general population. These regional differences have not been thoroughly studied.

While specific biochemical measurements and complementary tests have also been used to provide information on the sensitization potential of test materials, this report examines the methods which are more commonly performed.

2.3 Definitions

- Allergens are conventionally defined as substances that will induce immunologically dependent immediate or delayed reactions such as erythema, edema, and vesiculation, in subjects that have been previously exposed.

- Freund's Complete Adjuvant is a mixture of killed bacteria and mineral oil containing detergent. This is conventionally used to emulsify allergens in water.

- Sensitization reactions are frequently more severe and more persistent than irritation reactions, and sensitizers can be graded from "weak" to "strong" based on the number of subjects that become sensitized, rather than on the severity of the skin reaction.

A satisfactory predictive test for sensitization must (1) be able to detect weak sensitizers, (2) show a good correlation between positive and negative sensitizers in the test and known human sensitizers and non-sensitizers, (3) be reproducible, and (4) should be quick, easy to perform, and economical.

2.4 The Sensitization Test

A typical sensitization test involves exposing the subject animal to the test substance in order to induce a response to subsequent exposures; this phase is the induction phase (sensitizing). After a rest period of about 2 weeks (during which time the test subject develops the ability to respond to the test material) the subject is re-exposed. This is the challenge phase. The reaction during the latter phase is taken as evidence of sensitization only if no reaction in the control subject without induction is seen.

2.5 Methods for Sensitization

The salient features of the sensitization process are as follows:

(1) When a simple chemical substance (hapten) is applied to the skin it reacts with certain skin components. Two types of proteinconjugates - mobile and immobile - are formed at the site of application. The mobile forms are allergenic and penetrate to regional lymph nodes where after a suitable incubation period, a complicated immunologic process results in the production of sensitized lymphocytes. The immobile forms remain *in situ* for some time and react with the sensitized lymphocytes that are produced on reapplication of the hapten. This interaction results in macroscopic dermal/epidermal alterations typical of contact dermatitis (leucocyte chemotaxis, vasodilatation and increased vasopermeation).

Contact dermatitis in humans is characterized by itching, erythema, erythema, edema and vesiculation, and sometimes purpura and necrosis whereas in the guinea pig the main features of sensitization are erythema and edema. Characteristic histological features in humans include spongiosis, exocytosis, vesicles, bulla formation, pustules, necrosis, acantholysis of the epidermis and perivascular infiltration, eosinophilic leucocytes, dilatation of the lymphatic vessels and blood capillaries and edema of the dermis (Nater and Hoedemaeker, 1976).

(2) A second requirement for skin sensitization is that the potential allergen must gain entrance into the body. To enhance penetration of the sensitizer, the skin site must be clipped, shaved or pretreated (generally with sodium lauryl sulfate), irritated, excoriated (with sand paper), stripped (with tape) or frozen.

A mixture of dead mycobacteria in paraffin oil is often used in the guinea pig for its adjuvant properties. Mycobacteria in oil and picrylated stromata, for example, can be included in an injection by using an emulsifier, such as Freund's Complete Adjuvant.

(3) When the skin is tested for hypersensitivity, the results obtained may differ due to the route of administration of the test material (topical application or intradermal injection). The topical route (epicutaneous) is the natural route of entry of most contact allergens, but the intradermal route offers a more rapid entry to the lymphatic system and a higher concentration can be achieved by this method than by the epicutaneous route. The reactivity of the skin may vary with the season and the test results may be affected by the degree of intactness, hydration, contact pressure and occlusion.

(4) Other factors of importance during dermal sensitization testing are the physicochemical properties of the vehicle, concentration of the test substance, and duration of the exposure. Additionally, genetic background, age, pregnancy and general state of health of test animals are important variables (Magnusson and Kligman, 1977; Coenraads _et al_., 1975).

2.6 Experimental Animal

The albino guinea pig is used for dermal sensitivity testing because it most closely resembles man in its response to sensitizers (See Section 6.0). Young adult animals (300-500 gm) of both sexes and various strains (Hartley, Pirbright, or Himalayan white-spotted) with proven allergenic aptitude are generally used. In many studies, a positive control group of animals is exposed to a known sensitizer in order to demonstrate allergenic aptitude.

3.0 PREDICTIVE TEST METHODS FOR SKIN SENSITIZATION USING GUINEA PIGS

In the cutaneous sensitivity tests developed in the last few years for the guinea pig, an attempt has been made to improve the correlation of results with human skin sensitivity compared to the intradermal test of Draize (1965).

As noted above the most common procedures currently used in the guinea pig for testing materials for contact dermatitis can be classified into three groups according to the method used to administer the allergen. They are -

- o Group I, Draize Test and the Freund's Complete Adjuvant Test which use the intradermal route,
- o Group II, The Open Epicutaneous Test and Buehler's Closed Patch Test in which the allergen is applied topically; and
- o Group III, which includes the Kligman-Magnusson guinea pig Maximization Test, the Optimization Test of Maurer, the Maguire Split-Adjuvant Test and the Footpad Technique of Roudabush, all of which use both the intradermal and epicutaneous routes.

The details of these test procedures are summarized and compared in Table 2-1.

3.1 Draize Test

This test is based on the classical Landsteiner technique (Landsteiner and Jacobs, 1935) as modified by Draize (Draize 1965).

Animals: Twenty guinea pigs each are used in the test and control groups for each test material.

Test Material: The test material is injected intradermally as a 0.1% solution (suspension or emulsion if solid or powder) in physiological saline, paraffin oil, or propylene glycol.

Induction: Induction is achieved by a series of 10 intradermal injections (one every alternate day) of the test material (0.05 or 0.1 ml) into different sites on the shaved anterior flank of the guinea pig. Skin reactions are read 24 hours after each injection.

Challenge: Challenge is performed 14 days after the tenth intradermal injection on the contralateral flank of the animal on a site corresponding to the site of the first injection. Control animals receive the same treatment with 0.05 ml of the 1.0% test solution given intradermally. Readings are taken 24 and 48 hours later on shaved skins to determine the intensity of erythema and size of edema of the test reaction. The reactions of all animals to the first intradermal injection (0.05 ml) are compared with reactions at challenge and with those in control animals. When a large variation is observed between the reactions within the same group, the mean values for induction and challenge phases of the test and control animals are compared.

Table 2-1. Predictive Tests For Guinea Pig Skin Sensitization[a]

Test	Draize[b]	Freund's Complete Adjuvant	Open Epicutaneous	Closed Patch	Maximization	Optimization	Split Adjuvant	Footpad
NO. ANIMALS								
Test/Vehicle Control	20 per group	8-10 per concentration	6-8 per concentration	10-20 per group and negative controls	20-25 per concentration	20 per group	10-20 per group	10 per group
INDUCTION								
Test Substance, amount or concentration	0.05 or 0.1 ml of 0.1% solution suspension or emulsion	0.1 ml of 5-50% in FCA	0.1 ml undiluted or concentrations of 30, 10, 3, 1% or lower	0.5 ml MIC	0.1 ml varied concentrations alone and in FCA	0.1 ml of 0.1-10% test substance in FCA	0.2 ml ointment or 0.1 ml liquid (varied concentrations) and 0.1 ml FCA	0.05 ml of 1.0% (W/V) in FCA
Vehicle	Physiological saline, paraffin oil or PG	Water, acetone, Ethanol PG, petrolatum	Same	Ethanol, acetone, tetrapropylene benzene sulfonate	Water, paraffin oil, peanut oil, PG	Physiological	Various solvents	
Skin Site	Anterior flank (shaved)	Flank (shaved)	Flank (clipped)	Flank	Shoulder (clipped)	Flank and back	Back (shaved)	Front foot pad
No. Intradermal Injections	10; on alternate days; observe 24 hrs after each injection	5; on alternate days; observe 24 hrs after each injection	NA	NA	6; 3 pairs made simultaneously on day 0	9; 3 pairs made over 3wk; the last 6 being made together with FCA	1 FCA only, prior to 3rd induction patch	1
No. Epicutaneous Applications (Open.).	NA	NA	21; consecutively to same site or 5 times a week for 4 weeks	NA	NA	NA	NA	NA
Type Patch	NA	NA	NA	7/8x1" webril occluded with elastoplast coverlet	2x4 cm filter paper occluded with impermeable plastic adhesive tape secured with adhesive bandage	NA	2x2 cm; filter paper occluded with impermeable plastic adhesive tape secured with adhesive bandage	

Table 2-1. Predictive Tests For Guinea Pig Skin Sensitization[a]

(Continued)

Test	Draize[b]	Freund's Complete Adjuvant	Open Epicutaneous	Closed Patch	Maximization	Optimization	Split Adjuvant	Footpad
No. Patches	NA	NA	NA	3 days 0, 7 and 14	1 1 week after injection	NA	4	NA
Patch Duration (hrs)	NA	NA	NA	6	48	NA	48 after each application	NA
REST (days)	14	11	0	14	14	14	10	7
CHALLENGE								
Test substance, amount and concentration	0.05 ml of 1.0% solution or suspension	0.05 ml MIC and MNIC	0.025 ml MIC and MNIC	0.5 ml MNIC[c]	0.1 ml MNIC[c]	0.1 ml of 0.1-10% solution	0.1 ml of varied concentrations	0.3 ml of 1% solution in fat: dioxane: acetone (1:2:7) solvent system
Skin Site	Contralateral flank	Same	Same	Flank (shaved, both sides)	Flank (shaved)	Flank (untreated fresh site; shorn)	Back (clipped)	lower back (clipped)
No. Intradermal Injections	1; observe 2 days					1; Test material without adjuvant	NA	
No. Epicutaneous applications (open)	NA	2; on days 21 and 35	Same	NA	NA	NA	NA	1
Patch Type	NA	NA	NA	Same as induction	2x2 cm filter paper - same as induction	2x2 cm filter paper covered by occlusive plastic foil	Same as induction	Same
No. Patches	NA	NA	NA	1	1	1 14 days after ID challenge	1	NA

Table 2-1. Predictive Tests For Guinea Pig Skin Sensitization[a]

(Continued)

Test	Draize[b]	Freund's Complete Adjuvant	Open Epicutaneous	Closed Patch	Maximization	Optimization	Split Adjuvant	Footpad
Patch Duration (hrs.)		24 (open); observe 3 days	24 (open) observe 3 days	6 observe 18 hrs later	24 observe 2 days	24 observe 24 hrs later	24 observe 2 days	24 (open) wash site observe 3 hrs later

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[a] Abbreviations:
FCA = Freund's complete adjuvant
NA = Not applicable
PG = Propylene glycol
MIC = Minimal irritating concentration
MNIC = Maximal nonirritating concentration

[b] Reference for test methods:
Draize - Draize, 1965
Freund's Complete Adjuvant - Klecak et al., 1977
Open Epicutaneous - Klecak et al., 1977
Closed Patch - Buehler and Griffith, 1975
Maximization - Magnusson and Kligman, 1969
Optimization - Maurer et al., 1975a
Split Adjuvant - Maguire, 1973
Footpad - OECD, 1981

[c] If test agent is a nonirritatnt, the test site is pretreated with sodium lauryl sulfate in petrolatum 24 hours prior to challenge to enhance penetration of test material.

If the challenge values are substantially higher than the erythema/edema values noted during induction or in control animals, the substance is considered to have produced sensitization. The degree of sensitization is proportional to the increase in the final reading compared to the mean of the readings following the 10 original doses.

Evaluation and Critique: The Draize Test is easy to perform, economical, and useful as a screening method for detecting "strong" and "moderately strong" sensitizers. However, the route of application (intradermal) does not parallel normal human exposure; the induction concentration fixed at 0.1% does not relate to the use concentration; reaction readings are not easily quantifiable; it is not appropriate to detect "weak" or "moderately weak" sensitizers and cannot be used for testing many finished bacteriostatic products, due to the presence of irritating bacteriostats, stabilizers, pigments, and other ingredients. Because of its limitations, the Draize Test has been frequently modified or replaced by other testing techniques in an attempt to improve the sensitivity (Prince and Prince, 1977). In several instances, the sensitivity of this test has been enhanced by simply increasing the number of injections and raising the dose of the test material.

3.2 Freund's Complete Adjuvant Test

The Freund's Complete Adjuvant test (Klecak et al., 1977) is a variant of the intradermal test method. It is a semiquantitative test method in which the sensitizing concentration and the minimal eliciting concentrations (i.e., epicutaneous) are determined by the investigator.

Animals: Eight to 10 guinea pigs each are used in the test and control groups at each concentration of the test material.

Test Material: For induction, the test material is incorporated in Freund's Complete Adjuvant so as to give a final concentration range of 5-50%. For the challenge, the test material is diluted, emulsified or suspended in an appropriate vehicle (water, acetone, ethanol, propylene glycol, petrolatum).

Induction: Induction is achieved by intradermal injection of 0.1 ml of varied concentrations of the test material in Freund's Complete Adjuvant into the shaved flank of the guinea pig skin on alternate days (5 times in all) in a 3 x 2 cm area. The control animals receive 0.1 ml of Freund's Complete Adjuvant alone.

Determination of the Threshold Concentrations: One day prior to start of challenge, varied concentrations (3-100%) of the test agent are topically applied in 0.025 ml portions to the clipped left flank skin of four untreated guinea pigs ($2\ cm^2$ area) simultaneously. The application site is left uncovered, and skin reaction is read 24 hours later. The maximal nonirritating concentration (MNIC) and the minimal irritating concentration (MIC) are determined. The minimal irritating concentration is defined as the lowest concentration causing mild erythema in 25% of the treated animals; maximal nonirritating concentration is defined as the highest concentration which induces no macroscopic reaction in any animal.

Challenge: On the 12th day after the last intradermal injection, i.e. on day 21 and again 14 days later on day 35, 0.025 ml of the minimal irritating concentration and maximal nonirritating concentration are applied to the contralateral flank of the test and control animals. The application is made over a 2 cm^2 skin area which is left uncovered. Reactions are read at 24, 48, and/or 72 hours after the application. The test material is considered allergenic when 1/8 of the animals tested show a positive reaction to the maximal nonirritating concentration used at challenge.

Evaluation and Critique: This test is technically simple and economical, but it is not adequate for testing finished products and often gives false positive results with materials found later to be negative in the Maximization test in humans. It is, therefore, useful as a screening test in the guinea pig but is a poor predictor of human response (Klecak et al., 1977).

3.3 Open Epicutaneous Test

The open epicutaneous (no patch) test has been proposed for testing the skin irritant and allergenic capacity of chemical agents intended for use in cosmetics, perfumes, and dermatological products (Klecak et al., 1977). It has not been tested on industrial chemicals.

Animals: Six to eight guinea pigs each are used in 1 to 6 test groups along with 1 control group.

Test Materials: The test materials are used undiluted or dissolved, suspended or emulsified at concentrations of 30, 10, 3, and 1% or lower in a suitable vehicle (acetone, water, ethanol, propylene glycol, petrolatum, etc.). Application volumes are held constant.

Determination of the Threshold Concentrations: One day prior to induction a test group is used to determine the maximal nonirritating and minimal irritating concentrations of the test material using the method described under Freund's Complete Adjuvant test-challenge.

Induction: Induction is achieved by application of 0.1 ml portions of undiluted test agent and its progressive dilutions to the clipped flank skin (8 cm^2) of the guinea pig. Application is repeated daily for 3 weeks or 5 times weekly for 4 weeks using the same site. The application site is left uncovered and the reaction is read 24 hours after each application. The maximal nonirritating and minimal irritating concentrations are determined by the all or none criterion. When the reaction is strong, the application site is changed.

Challenge: Challenge is made on day 21 (on the day of last induction) and 35 (14 days after last induction). A 0.025 ml portion of the minimal irritating and maximal nonirritating concentration of test agent is applied to the contralateral flank skin (2 cm^2); the application is left uncovered, and the reaction is read after 24, 48, and/or 72 hours. Vehicle treated or untreated (negative control) guinea pigs are treated likewise and the reactions read at similar intervals. The minimal irritant concentration of the test agent is used to confirm the biological activity determined before starting the induction and to exclude false results due to instability of the agent.

This procedure permits determination of the minimal sensitizing concentration necessary to induce allergenic contact hypersensitivity and the minimal eliciting concentration necessary to cause a positive reaction. A concentration is considered allergenic when at least 1/8 of the animals of a group show a positive response.

Evaluation and Critique: The open epicutaneous method of testing employs several concentrations of the test material at one time in contrast to most of the other procedures. It is realistic and appears to be in accord with the current needs of the pharmaceutical and cosmetic industries in that (1) substances are tested by repeated uncovered topical application, which parallel use conditions, and tests are easy to perform; (2) the test material is used in decreasing concentrations to establish a dose response curve; and (3) the test is scored on an all-or-none basis, considering the use concentration.

The open epicutaneous method for testing for allergenicity is suitable for testing mixtures and finished products as no injection is involved.

3.4 Closed Patch Test

Buehler (1964, 1965), and Griffith and Buehler (1977) have presented several predictive tests using the guinea pig which have introduced the use of a patch in the application of substances.

The experimental evaluation of skin sensitization in guinea pigs according to these authors (Buehler and Griffith, 1975) is as follows:

Animals: Ten to twenty guinea pigs each are used in the test, vehicle control, and negative control groups. The vehicle controls are sometimes omitted.

Test Materials: The test agent is diluted, emulsified, or suspended in a suitable vehicle (ethanol, acetone, tetrapropylene benzene sulfonate etc.). The concentration used during induction is one which causes skin irritation and is obtained from the concentration of use.

Induction: Induction is evoked by application of 0.5 ml portion of the test material at the minimal irritating concentration to the flank skin and held in contact by an occlusive patch for 6 hours. The vehicle control group is treated likewise except only the vehicle is applied. During the exposure, the animals are immobilized in a special restrainer. The patching is repeated on days 7 and 14.

Challenge: Fourteen days later, the animals are challenged using the 6-hour occlusive patch test of the induction phase. The maximum non-irritating concentration is used. The patch is applied to shaved flank skin on both sides in the test group but only on the left side in the two control groups. The test group is challenged with the test material in the vehicle, the vehicle control group with the vehicle alone, and the negative control group (not pretreated in the induction phase) with the test material in the vehicle.

Eighteen hours after patch removal, the flanks are depilated and the reaction is read. Readings are again taken at 48 and 72 hours after patching. The reaction is graded on a scale of 1 (slight erythema) to 3 (marked erythema). The results are expressed in terms of the incidence and severity of response. Incidence is derived from the number of animals showing response at 24 or 48 hours divided by the number tested. Severity is calculated from the sum of the test grades divided by the number of animals tested.

Evaluation and Critique: The closed patch test of Buehler and Griffith (1975) has all the essentials of a predictive test: the applicaton is by the topical route; finished products can be tested as such; the induction concentration can correspond to user concentration; the vehicle chosen can be similar to the end formulation and the sensitivity of the method is comparable to the repeated insult patch test for humans. A subjective factor involved in the evaluation of the severity of the response could bias the conclusion.

Comments: There are numerous variants of this test in existence today but their use has been described as limited (Maurer et al., 1975a).

3.5 Maximization Test

Magnusson and Kligman (1969, 1970) and Magnusson (1975) developed a procedure in the guinea pig for identifying contact allergens in which a deliberate attempt is made to "maximize" the chance of inducing sensitization without regard to realistic skin exposure. This test combines the use of the intradermal injection and the patch. It is considered to be the most sensitive test currently in use. Results from these tests have shown a high degree of correlation with similar tests in humans (Kligman, 1966c) which are described in Section 5.4.

Animals: Twenty to 25 guinea pigs each are used in the test and control groups.

Test Material: The test material is applied intradermally and epicutaneously during induction and epicutaneously at challenge. For injection, water-soluble test agents are dissolved in the water phase and emulsified in Freund's complete adjuvant; oil-soluble or insoluble materials are dissolved or suspended in the adjuvant (a mixture of paraffin oil and an emulsifier with mycobacteria). Paraffin and peanut oil, or propylene glycol are used to dissolve or suspend water insoluble materials which are to be injected without adjuvant. When solids are used for topical application they are ground to fine powder and incorporated in petrolatum. Liquid materials are applied neat (if not irritating) or in dilutions in petrolatum or water. If the test agent is an irritant, a concentration which causes a weak to moderate inflammation is selected for use.

Induction: Induction is achieved in two steps. On day zero, 3 pairs of intradermal injections are made simultaneously to a clipped skin site on the shoulder (3 injections on either side of the midline) within a 2 x 4 cm area. Injections are: (1) 0.1 ml Freund's Complete Adjuvant alone, (2) 0.1 ml test material alone, and (3) 0.1 ml test agent in Freund's Complete Adjuvant. Control animals are treated likewise except the test material is omitted. On day 7 a filter paper patch (2 x 4 cm) spread with a thick, even layer of the test agent (or saturated, if liquid) is applied over the same shoulder area (after clipping) and secured by an impermeable plastic adhesive tape and bandage (occlusion). The exposure is for 48 hours.

If the test agent is a nonirritant, the test site is pretreated with sodium lauryl sulfate in petrolatum 24 hours prior to patching, to enhance penetration. Otherwise, the allergen is applied at concentrations which produce irritation. Control groups are treated similarly with the test agent.

Challenge: Challenge is performed on the shaved flanks after a further 2-week period (day 21). Occlusive patches (2 x 2 cm) spread or saturated with the maximum nonirritant concentration of the test agent are applied for 24 hours to the right (patch with vehicle) and left (patch with test agent) sides. Kero and Hannuksela (1980) used aluminum chamber units instead of the patches, and because of the small size of the units, were able to perform all the challenges on the same flank.

Readings are made 24 and 48 hours after the removal of the patches and cleansing of the test site. Reactions are scored on a 4-point scale with 1 (one) representing mild and 4 representing extreme redness and swelling. The important statistic in the maximization test is the frequency of sensitization and not the intensity. The rating is based on the percentage of animals sensitized; test substances are assigned to one of five classes:

MAXIMIZATION GRADING IN THE GUINEA PIG

Sensitization Rate (%)	Grade	Classification
0-8	I	Weak
9-28	II	Mild
29-64	III	Moderate
65-80	IV	Strong
81-100	V	Extreme

(Magnusson and Kligman, 1969)

Evaluation and Critique: The Magnusson-Kligman (1969, 1970, 1975) Maximization method of testing virtually eliminates the false negative results of the Draize (1965) technique. It is an excellent procedure for identifying "weak" contact allergens. The method is, however, less adequate for predicting sensitization to finished products as intradermal injection of irritating components is involved.

The treatment with either Freund's Complete Adjuvant or the occlusive bandage may lower the threshold level for skin irritation. As such, a challenge concentration of the test agent, which in untreated animals is found to evoke no response, might be active in an animal treated with adjuvant in an occlusive dressing. In this situation, a false positive response would be recorded. To exclude false positive results, the control group should be treated the same way as the animals in the test group.

Comments: Several variations of the Maximization test procedure have been tried (Fahr and Schulz, 1976) and found to be less sensitive than the Magnusson-Kligman procedure described above.

3.6 Optimization Test

Maurer and his colleagues (Maurer et al., 1975a, 1975b, 1978, 1980) have recently developed an alternative method to the Maximization Test involving additional Freund's Adjuvant and have called it the Optimization Test.

Animals: Twenty guinea pigs each are used in the test and control groups.

Test Material: The test materials are used in varied concentrations (0.1-10%). Water-soluble substances are dissolved in 0.9% NaCl and mixed with Freund's Complete Adjuvant; substances soluble in oil are dissolved in Freund's Complete Adjuvant and mixed with 0.9% NaCl. The 1:1 mixture of adjuvant and saline are prepared shortly before administration.

Induction: Induction is achieved over a 3-week period. First week: 0.1 ml of the test substance is injected intradermally in to the flank and back (day 0), and into the back twice (days 2 and 4). Twenty-four hours later, the sites are chemically depilated (5 min) and 3 hours later the reactions are assessed. The two largest diameters of the erythematous reaction in vertical alignment are measured and the skin-fold thickness (mm) determined with a skin-fold gauge. From these values the individual "reaction volume" (microliter) is calculated for each animal and each reaction. Second and third weeks: intradermal injections of 0.1 ml of the test agent in Freund's Complete Adjuvant are made into the nuchal skin as for the first week.

Challenge: Challenge is performed in two steps.

First challenge: Fourteen days after the last induction dose, the test material is injected in the same dose and volume as in the first week of induction (without Adjuvant) at a fresh site on the untreated flank. Twenty-four hours later, the reaction is measured and the reaction volume determined as during the first week of induction.

Assessment: The average extent of the reaction to the first four induction doses (first week) and the standard deviation are calculated for each animal. The mean and standard deviation of the four induction doses are then added to give an individual threshold value for each animal. If the reaction volume at challenge exceeds the corresponding threshold value, the animal is considered to be sensitized. The number of positive animals in each group is counted and the significance of the differences between the treated and control groups is assessed by the Fisher Exact test.

Second challenge: A further epidermal challenge is administered fourteen days after the first challenge. The test substance in the appropriate vehicle (to give the maximum nonirritant concentration) is spread uniformly on a patch (2.0 x 2.0 cm) of filter paper, which is applied to a shorn, untreated site, covered with occlusive plastic and left in place for 24 hours. Twenty-one hours after patch removal, the site is chemically depilated and 3 hours later the extent of erythema and skin-fold thickness is determined.

Assessment: The presence of clearly discernible reddening of the reaction site is taken as indication of an allergic reaction. The significance of the difference in the number of positive animals in the treated and control groups is assessed by the Fisher Exact test.

Evaluation and Critique: The Optimization Test appears to be more efficient than the Draize Test in detecting sensitizers, and comparative studies show that there is good agreement between the Optimization Test and the Maximization Test and between both tests and experience in man. In the Optimization Test the element of subjectivity is significantly reduced by using the reaction volume as the main evaluation criterion. Also the measurement of reactions occurring during the first week of induction, the calculation of standard deviations and the comparison in the same animal help assure that the effective reaction is objectively assessed.

With reference to the importance of the two challanges the authors indicate that "the first intradermal challange (with the same dose and volume of substance that is used in the initial phase of induction) is usually appropriate to elicit a positive reaction if sensitization has taken place. The second epidermal challange (under occlusion), serves only as an added precaution that takes further kinds of affinity of the test substance for the skin into consideration." (Mauer et al 1975a)

3.7 Split-Adjuvant Test

In a series of articles published between 1972-1975, Maguire and his coworkers (1972, 1973, 1975) described an adjuvant technique that amplifies the sensitization process in the guinea pig to allow detection of "weak" and "moderately weak" contact allergens in humans. This technique derives from the split-adjuvant test procedure (Maguire and Chase, 1967, 1972) in which the allergen and Freund's Complete Adjuvant are administered separately and together with the test material (Maguire, 1973) rather than as an emulsion. It is based on the observation of Magnusson and Kligman (1970) that intradermal injection of Freund's Complete Adjuvant beneath a site where a topical allergen has been applied for challenge, greatly potentiates the sensitization.

Animals: Ten to twenty guinea pigs each are used in the test and control groups.

Test Material: The test material is used at varied concentrations as solutions or suspensions in appropriate solvents.

Induction: Induction is achieved in four steps: (1) on day zero, 0.2 ml of ointment (or 0.1 ml of liquid) of the test agent is applied topically to the shaved skin of the back and this is covered with filter paper and occluded with tape; (2) a second application is made under occlusion 48 hours later (day 2); (3) on day 4, the patch is removed and 0.1 ml of Freund's Complete Adjuvant is injected intradermally twice into the sensitizing site followed by a topical application of the test substance under occlusion; and (4) on day 7, the patch is removed and the test material reapplied under a closed patch, which is finally removed on day 9.

Challenge: Challenge is performed on day 20 by a closed patch test using 0.1 ml of the test agent; the patch is applied to the clipped skin of the back. Twenty-four hours later (day 21), the patch is removed and readings are made. Further readings are made at 48 and 72 hours (day 23 and 24). Retesting at a different site for a second or third time occasionally will bring out borderline earlier readings.

The intensity of the reaction is classified as follows:

0 = Normal skin
± = Very faint, nonconfluent pink
+ = Faint
++ = Pale pink to pink, slight edema
+++ = Pink, moderate edema
++++ = Pink and thickened
+++++ = Bright pink, markedly thickened

Sensitization is assessed by comparing the number, intensity, and duration of skin reactions in the test and control groups.

When repeated contact applications are made during induction, the degree of sensitivity of the animal to low concentrations of the allergen rises stepwise with the concentration range. This heightening of reactivity to the allergen represents an anamnestic response in the area of delayed hypersensitivity and contrasts sharply with relatively high antibody titers found when chemical allergens are incorporated into Freund's

Complete Adjuvant and administered as a single injection (combination technique of Chase, 1954).

Evaluation and Critique: The Maguire Split Adjuvant Test method has all the features of a predictive test. Finished preparations can be tested as such and other substances can be tested with regard to the concentration of use. The repeated exposure and the number of patches applied during induction qualifies this procedure as a test for detecting weak sensitizers. It could also be used for screening of numerous chemicals, in which the dose dependence of presumed allergenic properties is to be checked, but this is cumbersome. The use of a different but still subjective scoring method is less appropriate than the more quantal methods developed recently. To exclude false positive results, it is important that the control animals be pretreated with Freund's Complete Adjuvant.

Comments: Several variations of the Maguire Split Adjuvant Test technique exist; however, their use is limited (Fahr and Schulz, 1976). One variant involving sensitization through intraperitoneal injection of pre-formed stromata conjugates in Freund's Complete Adjuvant can only be used with chemicals that couple with red blood cell stromata (Chase, 1954, 1967).

3.8 Footpad Test

The footpad technique for evaluating sensitization potential in the guinea pig (OECD 1981) is as follows.

Animals: Ten guinea pigs each are used in the test and control groups.

Test Material: The material is incorporated into Freund's Complete Adjuvant to give a 1.0% mixture (w/v). It is stirred gently at room temperature for 3 hours and is allowed to settle for a few minutes prior to being used.

Induction: Induction is achieved by injection of 0.05 ml of the test mixture into the front footpad of the guinea pig, which has not been previously exposed to the test material by any route. Controls are injected with Freund's Complete Adjuvant instead of the test material.

Challenge: One week later, 0.3 ml of a 1.0% mixture of the test agent in a solvent system of guinea pig fat:dioxane:acetone (1:2:7) is applied to the clipped skin of the lower back of the guinea pig. If a 1.0% solution produces moderate irritation, a 0.1% solution is used. Controls are challenged similarly.

Twenty-four hours later, the area is depilated, washed (with warm tap water, 37°C) and three hours after this the reaction is scored under fluorescent light for erythema and edema. The reaction is graded on a scale of 1 (slight erythema) to 4 (dark red, with hemorrhagic areas accompanied with swelling and increased skin temperature). The degree of swelling of the skin is determined by lifting the skin fold (about

1 cm in length), feeling it (between thumb and forefinger), and grading on a scale of 1 (slight, just detectable) to 3 (marked, difficult to pick up a fold). The individual scores of the two reactions for each animal are added and the mean value derived. The means of test animals are then compared with those of the controls which are similarly derived.

The difference in the reaction values between the control guinea pigs (primary irritation) and the test guinea pig is considered to be a measure of the degree of skin sensitization.

Preparation of Guinea Pig Fat: The following method has consistently yielded adequate product:

1. Strip fat from large, obese guinea pig.
2. Freeze.
3. Grind frozen fat (kitchen-style meat grinders).
4. Add acetone to ground fat (10:1, acetone:fat). Stir and heat over a hot water bath.
5. Filter (Whatman #4).
6. Add activated charcoal and stir (warmed).
7. Filter through Whatman filter #4 and Supercel. Pour into beaker.
8. Freeze (fat will solidify on the bottom of beaker).
9. Decant acetone and discard.
10. Gently warm fat and vacuum distill off acetone.
11. Pour warm fat into vials (use 1 vial per group of sensitizations).
12. Freeze all vials and use as needed (frozen fat will remain acceptable for at least a year).

Depilatory: The preparation and use of the following depilatory is suggested. Take 6 parts soluble starch, 6 parts talc, 6 parts barium sulphide, and 2.7 parts of granular nonirritant anionic surfactant. Add the ingredients in the order listed, mix well, and add cold water to make a viscous paste. Apply to clipped skin of guinea pig for 4 minutes. Rinse well with tap water.

Evaluation and Critique: No data are available in the open literature on this technique to enable adequate evaluation of its predictive nature.

Comment: At least one variation of this procedure is reported in the literature (Loomis, 1978) which differs sufficiently to warrant some explanation. Induction is achieved by topical applications on days 0 and 2 to the depilated back of the guinea pig, and reactions are read 24 and 48 hours after the first treatment only. After a rest period of 3 weeks, a challenge dose is applied to the depilated shoulder skin and reactions are read at 24 and 48 hours. The reaction scores made at induction and challenge are averaged for each group and compared. If the challenge scores are 2-4 times the induction scores, the test material is considered a moderate sensitizer; if the challenge score is 4-7 times higher, the agent is considered a strong sensitizer.

4.0 COMPARATIVE RESULTS OF SENSITIZATION TESTS IN THE GUINEA PIG

The sensitivities of different induction methods have been widely investigated with the view to determine the most appropriate mode of induction based on objective analysis and scoring of the results.

The effectiveness of different methods of sensitization is illustrated below with reference to various concentrations of a single compound or fixed concentrations of different compounds by three different routes of administration. The closed patch technique is more sensitive than the intradermal or epicutaneous route as sensitization was detected at lower concentrations or at a higher incidence with this technique.

Comparison of Methods for Induction of Sensitization in the Guinea Pig

Compound	Concentration	Incidence % Closed Patch	Incidence % Intradermal	Incidence % Epicutaneous
1-Chloro-2,4-dinitrobenzene	0.01	40-50		
	0.05	100	0	
	0.10			0
	0.25		30	
	0.5			70
p-Phenylenediamine HCl	2	100	0	--[a]
Tetrachlorosalicylanilide	1	80	0	--
Thioglycerol	14	60	0	--
Monobenzyl ether of hydroquinone	5	60	0	--
Formalin	5	30	10	--
Potassium chromate	1	10	10	--

[a] Not tested by this method
Data are from Griffith and Buehler, 1977.

The relation between dose and effects in contact allergy has been studied experimentally and by field survey. Results of these studies indicate a dose-dependent relation between induction and sensitization by intradermal as well as topical application (Griffith and Buehler, 1977, Matsushita and Aoyama 1980). The table below shows the responses to intradermal and epicutaneous (Closed patch) challenges in the guinea pig with different amounts of two unsaturated sulfones having 12 or 16 carbon atoms. The results show that the use of Freund's Adjuvant not only lessens the amount of sulfone required to induce sensitization, but also alters the relative sensitization potential of the C_{12} versus C_{16} sulfone as compared with the epicutaneous results.

The above results suggest that caution should be exercised in assigning relative sensitizing activity to topical agents that are tested intradermally with Freund's Complete Adjuvant when the two methods (intradermal or epicutaneous) are used as screening tests prior to human sensitization test.

Sensitization with 1-Alkenyl-1,3-sulfones in the Guinea Pig

		% Response to Sensitization, nmole[a]					
	Induction	Intradermal (in FCA)[b] Challenge			Epicutaneous (Closed Patch) Challenge		
Compound	nmole	7.1	71	710	71	710	7100
C_{12}	200	100	100	73.3	60	86.6	92.9
	20	100	78.6	46.6	26.6	53.3	71.4
	2	80	50	6.6	13.3	40	64.3
C_{16}	200	100	100	73.3	0	6.6	40
	20	93.3	86.6	0	0	0	6.6
	2	40	20	0	0	0	6.6

[a] Percent animals sensitized, nanomoles in challenge dose.
Data adapted from Griffith and Buehler, 1977.
[b] FCA = a Freund's Complete Adjuvant.

Maurer et al. (1975a, b), Magnusson (1975), and Magnusson and Kligman (1970) compared the sensitization potentials of several compounds in 3 different test methods (Draize test, Maximization test, Optimization test) and made the following observations (see Table 2-2):

(1) Potent allergens (e.g., dinitrochlorobenzene, DNCB) are readily assessed by the Draize test but this test is not sensitive enough to detect the so-called "weak" allergens (e.g., Penicillin G, formalin, ethylaminobenzoate).

(2) The known allergens (DNCB, Penicillin G, formalin, etc.) give significantly positive results with both the Maximization test and the Optimization test while the essentially non-allergenic (e.g., penicilloylpolylysine, paraffin, physiological saline, 5% ethanol) do not respond.

(3) "Weak" allergens responded equally well to both Maximization test and the Optimization test but not to the Draize test (e.g., formalin, CPY-1, CPY-2, Pencillin G, ethylaminobenzoate).

(4) Maximization test and the Optimization test sensitization rates are similar when the concentrations used for induction are the same (e.g., Penicillin G).

(5) Nickel sulfate experiments gave positive results with both the Optimization test and the Maximization test but no animals were sensitized with the Draize test. A higher percent of animals were sensitized by the intradermal than by the epicutaneous route, and the

Table 2-2. Comparative Results of Sensitization Test In Guinea Pig[a]

	Draize Test	Optimization Test					Maximization Test			
		Concentration %					Concentration %			
Test Material	Sensitization Rate	Induction ID[c]	Challenge ID	Challenge EC	Sensitization[b] Rate ID	Sensitization[b] Rate EC	Induction ID	Induction EC	Challenge EC	Sensitization Rate
DNCB[c]	20/20	0.1	0.1	0.1	20/20	20/20	0.1	1.0	0.01	20/20
PPL[c]	0/20	6×10^{-5} M	6×10^{-5} M	50×10^{-5} M	0/20	0/20	6×10^{-5} M	50×10^{-5} M	50×10^{-5} M	0/20
Penicillin G	0/20	0.1	0.1	10	0/20	10/20				
		0.3	0.3	10	0/20	15/20	0.3	5.0	10	14/19
	7/20[d]						3.0[d]	5.0	10	20/20
Ethylaminobenzoate	0/20	0.1	0.1	5	0/20	0/20	0.1	25	5	1/20
		2.0	2.0	5	10/20	9/20	2.0	25	5	6/20
							2.0[d]	25	5	7/25
Formalin	0/20	0.1	0.1	2[e]	20/20	10/20	5	5	2[e]	9/20
	1/20[d]						5[d]	5	2	16/20
CPY-1[c]	0/20	0.1	0.1	0.5	19/20	17/20	0.1		0.5	20/20
CPY-2[c]	0/20	0.1	0.1	0.5	17/20	15/20	0.1		0.5	20/20
Nickel Sulfate	0/20	0.1	0.1	0.1[f]	20/20	12/20		1.0[h]	0.5	0/20
	0/20	0.1	0.1	0.5[g]	20/20	7/20		1.0[g]	0.5	7/20

[a] Data compiled from Maurer et al. 1975a,b;1978.

[b] Number of positive animals/total number of animals.

[c] Abbreviations:
ID = intradermal
EC = epicutaneous occlusive patch
DNCB = 1-chloro-2,4-dinitrobenzene
PPL = penicilloylopolylysin
Penicillin G = benzyl penicillin potassium salt
CPY-1 = 1-(3-chlorphenyl)-3-phenyl-pyrazoline
CPY-2 = 1-(3-chlorphenyl)-3-(4 chlorphenyl)-pyrazoline.

[d] Magnusson and Kligman, 1970, and Magnusson, 1975.

[e] EC (occluded); 2% in water on shaven skin.

[f] EC (open); 0.1% in 70% ethanol on shaven skin.

[g] EC (occluded); 0.5% in water on depilated skin.

[h] EC (occluded); 0.5% in water on shaven skin.

sensitization rate was greater when the compound was applied to the open shaven skin than when it was applied to the depilated skin occlusively in both the Optimization test and the Maximization test.

(6) Increasing the penetration of the test substances applied to the skin by pretreatment (croton oil or sodium lauryl sulfate application) (Table 2-3), use of occlusive dressings or by administration of high concentration of test compound has much less influence on the efficiency of induction than has stimulation of the immune system by means of an adjuvant (e.g., ethylaminobenzoate which depends solely on the intradermal induction concentration to attain successful sensitization in the maximization test, Table 2-2).

Kero and Hannuksela (1980) compared the sensitization results of various concentrations of neomycin and propylene glycol in separate groups of guinea pigs by the Maximization test, Open Epicutaneous Test and the Chamber test methods (Table 2-4), and found no difference in the rate of sensitization between the Maximization Test and the Chamber Test with petrolatum and propylene glycol. In the Open Epicutaneous Test, neomycin in propylene glycol sensitized more readily (45% response) than in petrolatum (25% response). The results from all three methods agreed well and were not significantly different from each other. In the Open Epicutaneous Test the influence of the vehicle was more apparent than in either of the other two tests. The authors recommend the Open Epicutaneous Test over the Maximization Test because it is a more "natural" situation for contact sensitivity.

Benzocaine sulfamylon cream and cinnamic aldehyde were tested by the Maximization test, Split Adjuvant test, Open Epicutaneous test (modified Maguire test) and Draize test (Prince and Prince, 1977). In this study the Draize test method was again found to have the lowest sensitivity. The authors graded the 4 tests in the following descending order of sensitivity: Split Adjuvant; Maximization Test and Open Epicutaneous Test; Draize Test.

A comparison of the results for 5 compounds (allyl isocyanate, thioglycerol, paraphenylenediamine HCl, potassium chromate and formalin) tested for allergenic potentials by the Draize and closed patch test showed the closed patch method to be clearly superior (Buehler, 1965).

Potassium dichromate, nickel sulfate and sodium zirconium lactate are established allergic sensitizing agents in man. The potassium and nickel compound produce a superficial eczematous reaction, the zirconium compound induces a deep nodular granuloma associated with epithelial cell infiltration. Turk and Park (1977) tested all three salts by the Maximization test, Split Adjuvant test and the "Polak" test and found that it took longer to sensitize the guinea pig to zirconium than to chromium or nickel. All three protocols produced a delayed hypersensitivity-like reaction to zirconium. The best protocol for chromium and zirconium sensitization was the "Polak" method and the most effective method for nickel was the Split Adjuvant test (Table 2-4). The reaction with all three metals was often transient, which was evident in the fact that animals that failed to show sensitivity on an earlier challenge would develop sensitivity at a later one. Cross reactivity was also noted between zirconium and chromium. It is apparent that the Maximization Test is not highly sensitive to metal salts.

Table 2-3. Percent of Animals Reacting Per Group in the Optimization Test, Maximization Test and the Epidermal Test[a]

Induction	Challenge (10ug DNCB/Appl.) Intensity of Erythema (%) Appl. 1	Appl. 2	Appl. 3
Optimization Group	20	55	90
Maximization Group	75	100	100
Epidermal test	5	45	45
Epidermal test + irritation with croton oil	55	45	95
Epidermal test + irritation with SLS	5	68	100
	Increase in skin thickness (%)		
Optimization Group	20	85	100
Maximization Group	5	63	90
Epidermal test	5	10	10
Epidermal test + irritation with croton oil	50	55	85
Epidermal test + irritation with Sodium lauryl Sulfate	5	63	100

[a] Data from Maurer et al. 1978.

Table 2-4. Comparison of Six Protocols For Development of Delayed Hypersensitivity to Seven Compounds[a]

Compound	Induction (Conc. %)	Challenge (Conc. %)	Positive Reaction at Challenge[b] (# of animals/total)					
			MT	OET	CT	SAT	DT[c]	PT[d]
Neomycin (in pet.)[d]	30	20	7/20	5/20	5/20	---	---	---
Neomycin (PG/70%)	30	20 (in pet.)	6/20	9/20	6/19	---	---	---
Neomycin (PG/70%)	30	70 (in water)	0/20	0/20	0/19	---	---	---
Benzocaine (in pet.)	5	5	6/20	11/20	---	17/20	3/20	---
Sulfamylon (emulsion)	5	5	9/20	8/20	---	16/20	2/20	---
Cinnamic aldehyde (in pet.)	2	2	16/20	15/20	---	20/20	4/20	---
Potassium dichromate	1 mg	0.3 mg	0/6	---	---	3/6	---	3/12
Nickel sulfate	1 mg	0.33 mg	0/6	---	---	2/5	---	0/6
Sodium zirconium lactate	1 mg	0.33 mg	0/6	---	---	0/6	---	2/4

[a] Data compiled from Kero and Hannuksela 1980 (Neomycin); Prince and Prince 1977 (Benzocaine, Sulfamylon and Cinnamic aldehyde); Turk and Parker 1977 (metals).

[b] Abbreviations - pet. = petrolatum; PG = propylene glycol; CT = Chamber test; MT - Maximization test; OET = Open Epicutaneous test; SAT = Split Adjuvant Test

[c] topical induction without irritation, with adjuvant (foot pad injection).

[d] Polak Test:

Day 0: 4 footpad injections of 0.1 ml of emulsion with 2 mg/ml of metal salts in Freund's complete adjuvant. In addition 0.1 ml of emulsion was injected into the nape of the neck (total dose 1 mg).

Day 14: Intradermal challenge by 25 micrograms of metal salt in 0.1 ml saline into shaved flank. This was repeated weekly for 13 weeks.

Klecak et al. (1977) selected 32 fragrances known to be allergenic to humans and tested them concurrently on separate groups of guinea pigs by the Draize test, Maximization test, Open Epicutaneous test and Freund's Complete Adjuvant test (Table 2-5). Twenty five of the 32 compounds were allergenic by one or more of these tests and 22 of these were detectable by the Open Epicutaneous test and 21 by the other tests. Thus 4 allergenic compounds were exclusively detected by the Open Epicutaneous test and 3 others solely by one or more of the three intradermal tests. On the basis of the number of positive responses, the Open Epicutaneous test was considered by the authors to be superior to the other three tests. The Draize test was again found to be the least sensitive of the four test methods.

Conclusions:

A review of the comparative data from various sources indicates that except for the Draize test all of the methods discussed above have some potential for detecting the so called "weak" sensitizers. No one test method appears to be suitable for testing of all compounds, and significant variations in results have been observed from different sources using the same test method, compound, concentrations (Table 2-2, e.g., Penicillin G, Maximization test).

Based on the published evidence, the guinea pig Maximization test is one of the better tests for detecting the weaker sensitizers, the use of which must be avoided in cosmetics and toiletries. The test virtually eliminates the false negative results seen with the Draize test but does not appear to be adequate for predicting sensitization to finished products. The Optimization Test, Open Epicutaneous Test and the Closed Patch Test may be viable alternatives but the published evidence on their reliability is insufficient. All three can be used on finished products. The Closed Patch test involves a subjective evaluation of the sensitivity, but allows testing of the actual user concentration. The Open Epicutaneous test is also flexible in this regard. Several of the commercial and industrial test labs contacted used both the Maximization and Closed Patch test with success.

The aim of animal testing is to establish to what extent a particular substance has the potential for acting as a skin sensitizer in humans. The tests reveal that a chemical possesses immunologic capabilities, but the percent of animals sensitized does not necessarily indicate the probable incidence of human sensitization. A negative result with a sensitive test method predicts with a reasonable degree of certainty that the test material will not be a sensitizer. On the other hand, this does not mean that the substance will never sensitize anyone but rather that the risk of sensitization to humans is low.

Table 2-5. Allergenicity of 32 Incriminated Compounds For Humans and Their Allergenicity For the Guinea Pig By Four Test Methods[a]

Group[c]	Total # of Cmpds.	DT[b]		MT		OET		FCA	
		pos	neg	pos	neg	pos	neg	pos	neg
I[d]	7	0	7	0	7	0	7	0	7
II	18	7	11	15	3	18	0	17	1
III	4	0	4	0	4	4	0	0	4
IV	3	1	2	3	0	0	3	3	0

[a] Data from Klecak et al. 1977.

[b] Abbreviations - DT = Draize Test; MT = Maximization Test; OET = Open Epicutaneous Test; FCA = Freund's Complete Adjuvant Test; pos = positive; neg = negative.

[c] Group I - Acetophenone
Benzophenone
Diethyl phthalate
Dimethyl-benzyl carbinol
Dimethyl benzyl phthalate
Hydroxycitronellal
Thymol

Group II - Benzyl alcohol
Benzyl cinnamate
Carvacrol
Cinnamic aldehyde
Citral
Citronellal
Cuminic aldehyde
Geraniol
Helitropin
Isoeugenol
Limonene
Methyl cinnamate
Methyl heptine carbonate
Methyl octine carbonate
Phenylacetaldehyde
Phenyl-ethyl salicylate
3 phenyl-propionaldehyde
10-Undecenal

Group III - Amyl salicylate
Benzyl salicylate
Bromo styrol
Methyl salicylate

Group IV - Benzaldehyde
Cinnamic alcohol
Vanillin

[d] The compounds are subdivided into 4 groups according to their allergenicity in the 4 animal tests, namely: Group I, not sensitizing in any test; Group II, sensitizing in the OET and in one or more of the other tests; Group III, sensitizing exclusively in the OET; Group IV, not sensitizing in the OET but sensitizing in one or more of the other tests.

5.0 HUMAN SKIN SENSITIZATION TEST PROCEDURES

The human patch test has evolved from a single patch test (Schwartz, 1960), to the Prophetic Patch Test (Schwartz and Peck, 1944), the Repeated Insult Patch Test (Shelanski and Shelanski, 1953; Draize, 1965; Marzulli and Maibach, 1973, 1974; Maibach and Epstein, 1965) and finally to the Maximization test of Kligman (1966a,c). The human patch test should never, however, be used prior to screening a compound for activity in guinea pigs.

The procedures currently used for the Maximization test require repeated occluded patches during induction (10 patches, 48 hours each, same site) followed by a 2-week rest period, and a challenge. There are several variations which include the use of provocative chemicals (sodium lauryl sulfate) (Kligman 1966d), special skin preparation (freezing) (Epstein et al., 1963), high concentrations of test substance during induction (Marzulli and Maibach, 1974), special patches (Magnusson and Hersle, 1965) and increased number of test subjects (Kligman, 1966c). Methods of historic and current interest are summarized in Table 2-6.

The following are the most frequently used procedures for testing sensitization in humans.

5.1 Schwartz and Schwartz-Peck Test

The Schwartz (1960) and Schwartz-Peck tests (1944), described in Table 2-6 and later modified to include a 6-day induction period under occlusive conditions, is useful in testing polymeric materials which contain small amounts of low molecular weight sensitizing or irritant chemicals which are released from the polymer slowly. The Schwartz (1960) and the Schwartz-Peck test have certain inherent defects. False positives may occur since borderline primary irritants can sometimes produce reactions which can be confused with sensitization when only one patch reading is made; but more important are the false negatives which result from the single application of small amounts of the test substance, which is often inadequate to produce sensitization except in the case of strong allergens. The modified Use Test (see below) recommended by these authors was probably a recognition of this inadequacy.

5.2 Shelanski-Shelanski Test

Because the original Schwartz-Peck test with one 48 hour induction exposure was ineffective in testing cosmetics, Shelanski and Shelanski (1953) recommended multiple insults, involving 10-15 24-hour treatments on alternate days. This repetitive application to the same site gives a higher yield of sensitized subjects but tends to magnify the irritant properties of the test substance. The resulting "skin fatigue" due to repeated irritation under occlusive conditions could make the differentiation between irritation and sensitization difficult.

5.3 Draize Test (and modifications)

Marked by multiple insults at induction (10-15 treatments of 24 hours each), this test is extensively used for determining human sensitization

Table 2-6. Predictive Patch Tests For Human Skin Sensitization

Test	Schwartz	Schwartz-Peck	Shelanski-Shelanski Repeated Insult	Draize Repeated Insult	Draize Modified	Kligman "Maximization"	"Maximization" Modified
No. Subject	200	200	200	100 males, 100 females	200	25	25
INDUCTION							
Test substance, amount or concentration	Liquid or powder (on 1" fabric)	Liquid[a] (saturating ¼" 4-ply) gauze	Proportional to area of ultimate use; liquid or powder	0.5 ml or 0.5 g	0.2 ml or 0.2 g (high conc.)	1 ml 5% SLS followed by 1 ml 25% test material	1 ml 1% SLS[b] (24 hr) followed by 0.3 ml 0.3 g test material
Vehicle	None	Petrolatum or corn oil	None	NS[c]	Petrolatum	Petrolatum	Petrolatum
Skin Site	Arm, thigh or back	Arm or back		Arm or back	Arm	Forearm or calf	Forearm
Type Patch	Cellophane covered with 2"x2" Elastoplast	1" non-water proof cellophane covered with 2" adhesive plaster	Same	1" sq. band aid[R]	1" sq. band aid[R]; no perforations	1.5" sq. Webril occluded with Blenderm[R] held in place with perforated plastic tape	2 cm Webril occluded with Blenderm[R] held in place with perforated tape
No. Patches	1	1	10 - 15	10	10	5 same site	7
Patch Duration (hours)	72	27, 72 or 96	24 alternate days; same site	24 alternate days	48 - 72	24 hrs. SLS followed 10 days by 48 hr. test material for each of 5 inducing applications	24 hrs. SLS followed 10 days by 48 hr. test material for each of 7 inducing applications. No patch for 24 hrs. between each of 7 inducing applications
REST (Days)	7 - 10	10 - 14	14 - 21	10 - 14	14	10	10 - 14

Table 2-6. Predictive Patch Tests For Human Skin Sensitization

(Continued)

Test	Schwartz	Schwartz-Peck	Shelanski-Shelanski Repeated Insult	Draize Repeated Insult	Draize Modified	Kligman "Maximization"	"Maximization" Modified
CHALLENGE							
Test substance, amount or concentration	Same as induction	Same as induction	Same as induction	Same as induction	MNIC[c]	0.4 ml 10% SLS (1 hr.) followed by 0.4 ml, 10% test material	1 ml 2% SLS (½ hr.) followed by 0.3 ml or 0.3 g test material
Skin Site	Same site	Any site especially thin keratin	NS	New skin site	New skin site	Lower back or forearm	Upper back
Type Patch	Same as induction	Same as induction	Same as induction	Same as induction	Same as induction	1" sq. patch. Same as induction	Same as induction
No. Patches	1	1	1	1	1 (rechallenge if necessary)	1	1
Patch Duration (hrs)	72 observe 10 days	48 observe 3 days after patch removal	48 Reaction read	24 Same	72 Same	48 reaction read soon after patch removal and at 72 and 92 hrs	48 Same

---

[a] Modified for solids, powders, ointments and cosmetics. Concentration, amount, area and site of application are important in evaluating results. Authors recommend cosmetics be tested open (Schwartz and Peck, 1944).

[b] Sodium lauryl sulfate (SLS) pretreatment is used to produce moderate inflammation of the skin. SLS is mixed with test material when compatible. SLS is eliminated when test material is a strong irritant.

[c] Abreviations
MNIC = Maximal nonirritating concentration
NS = Not Stated

(Marzulli and Maibach, 1973, 1974, 1976a,b; 1980). Maibach and Epstein (1965) found that the sensitivity of the test could be increased by raising the concentration of the test substance.

Subject - 200 adult volunteers (who sign an FDA approved consent form before participation).

Test Material - The test material is used in graded concentrations (0.2 - 0.5 g) and at actual use concentrations (1/100 of the concentration producing a 0.1% response). The vehicle is generally petrolatum.

Induction - Sensitization is achieved by topical application of the test material in 0.2 ml portions to the skin of the upper lateral part of the arm. The site is covered with an occlusive patch (Band Aid^R with perforations) for 48 or 72 hours. Usually 10 epicutaneous applications are made successively to the same site.

Challenge - Challenge is performed 14 days after the last sensitizing application when the maximum nonirritating concentration of the test material is applied under occlusion to a fresh skin site for 72 hours. Reactions are assigned one of 4 grades, (1) erythema, (2) erythema with induration, (3) vesiculation, and (4) bulla formation.

Evaluation - The data collected over the years indicate that substances that are not strong sensitizers are not likely to give false positive reactions by this test, even when high concentrations are used at induction, provided that a non-irritant concentration is used for the challenge.

5.4 Maximization Test

The Maximization Test was first reported in 1966 (Kligman, 1966a,b,c,d), modified several times and updated in 1975 (Kligman and Epstein, 1975). It now takes into account the large variations and susceptibilities that exist between skins of different human populations and includes a pretest exclusion of subjects who are non-reactors to the test materials, hypersensitive to sodium lauryl sulfate or who are already sensitized by previous exposure to the chemical. A basic premise of the Maximization test is that the exposure be deliberately intensified to increase the chances of detecting weak allergens.

Subject - 25 adult volunteers (who sign an FDA-approved consent form before participation).

Test Material - The test material is 25% by weight in petrolatum (i.e., 25 gm compound to 75 gm petrolatum). If the agent is an irritant at this level, lower concentrations are used. If the agent is nonirritating it is used undiluted.

Pretesting - Before induction begins, all members of the test group are patch-tested (with the tested materials as they will be used at challenge - see below) to eliminate hypersensitive people and obtain a baseline reading for each material on each subject. The degree of reaction is recorded. This reaction must be appreciably exceeded following challenge before the patch reaction is considered allergic.

Induction - Induction is achieved by delivering 1.0 ml of 5% aqueous sodium lauryl sulfate to a Webril patch (1.5" square) and fastening this occlusively to the arm (with Blenderm tape) held in place with perforated plastic tape for 24 hours in order to produce a moderate inflammatory response. The same site is treated on the following day with a 48-hour (25% test material) occlusive patch with the test material. The 48-hour allergen patches are repeated for a total of seven exposures each, with no patch for 24 hours between each of the 7 inducing applications.

Challenge - Challenge is performed with 2% sodium lauryl sulfate for 30 minutes followed by a 48-hour occlusive patch with the test material to the skin of the upper back. Reaction is read soon after patch removal and at 72 and 96 hours after patching.

The reactions are graded on a scale of I to V based on the number of subjects sensitized as shown below:

HUMAN MAXIMIZATION TEST GRADING SCALE

Sensitization Numbers[a]	Grade	Classification
0-2/25	I	Weak
3-7/25	II	Mild
8-13/25	III	Moderate
14-20/25	IV	Strong
21-25/25	V	Extreme

[a] Kligman, 1966c.

Evaluation - The maximization procedure is useful in identifying very low-grade sensitizing chemicals following prescreening in the guinea pig. It is primarily designed to yield allergenicity ratings for individual substances, not complex mixtures, finished products or formulations. The number of substances tested by this method by the Research Institute for Fragrance Materials suggests that it is the most widely used human test method. A major problem with this procedure lies in the interpretation of false positive reactions resulting from "toxic" effects due to factors other than the test material (e.g., infection, pressure, miliaria, drug reactions, food allergies) (Kligman and Epstein, 1975).

It should be noted that the Maximization Test does not directly assess safety in use (except when negative) and it does not predict the incidence of sensitization in a population of users. A better estimate of sensitization potential might be obtained with 100-200 volunteers than with 25.

6.0 APPLICATION OF PREDICTIVE TESTS TO KNOWN CONTACT ALLERGENS

The validity of human and animal test methods for sensitization is determined by their ability to detect compounds which pose hazards to man. The most effective test procedures are those which provide reliable information on the degree of response to be expected.

6.1 Human Tests

The result of ten "moderately strong allergens" (whose allergenic activity was confirmed medically) tested by the Schwartz-Peck, Draize and Shelanski-Shelanski methods on groups of 200 volunteers are summarized in Table 2-7. Two of the ten allergens were detected by the Schwartz-Peck test, one by the Draize Test and four by the Shelanski-Shelanski procedure. None of the three test procedures detected penicillin G, streptomycin, neomycin and benzocaine (Kligman, 1966a).

When the Maximization Test was applied to those compounds, penicillin G and streptomycin produced grade I (weak) and grade II (mild) reactions at equivalent doses, while thephorin, tetrachlorosalicylanilide and monobenzyl ether of hydroquinone gave grade II and III reactions with one-half to 1/20th the concentrations used in the Schwartz-Peck, Draize and Shelanski-Shelanski Tests (Kligman, 1966b). The Maximization Test in humans is not only sensitive but highly reproducible as is evident from the data in Table 2-8. The reproducibility is extremely high at both ends of the scale; upon repetition, weak (grade I) and strong (grade V) allergens are likely to be assigned same rating. There is greater variability in the mid-zone (grades II-IV), however the shift is rarely more than one grade class (e.g., penicillin G; chloroquine diphosphate).

6.2 Guinea Pig Tests

When the system of grading of allergenic response in the human Maximization Test was extended to the guinea pig a good correlation was found between the results of the two tests (Klecak et al., 1977). Of the 22 sensitizing substances that were tested using this grading system in the human and the guinea pig, ten were within the same rating level; 7 were within a single grade level and 5 were within two grade levels of discrepancy. Seven weak allergens (e.g., vioform, vanillin, cinnamic alcohol, eugenol, geraniol, heliotropin, limonene) were recognized by the guinea pig test but not by the human test (Table 2-9).

In contrast to the above findings a comparison between the results of other tests, e.g., the human Repeated Patch Test and the guinea pig Closed Patch Test showed a wide variation in response between the two systems (Table 2-10) (Griffith and Buehler, 1977; Marzulli et al., 1968; Marzulli and Maibach, 1974).

Table 2-7. Predictive Patch Tests In Humans

Compound	Concentration^{a} %	Schwartz-Peckb^{b} Test	Draize Test^{b}	Shelanski-Shelanski	Maximization Test^{c}
Penicillin G	1.0	0/200	0/200	0/200	4/22^{d}
Streptomycin	1.0	0/200	0/200	0/200	8/22^{d}
Neomycin	.0	0/200	0/200	0/200	0/22^{d}
Benzocaine	2.0	0/200	0/200	0/200	1/22^{d} (5.0%)
Thephorin	5	2/200	2/200	6/200	2/23^{d} (0.1%)
Ammoniated Mercury	10	0/200	0/200	2/200	8/23
Tetrachlorosalicylanilide	0.2	2/200	0/200	9/200	6/25 (0.1%)
Monobenzyl ether of hydroquinone	20	0/200	2/200	9/200	7/23 (1.0%)
Furacin	0.2	0/200	0/200	0/200	0/22^{d}
Butyn-Sulfate	2.0	0/200	0/200	0/200	--

[a] Concentration same for induction and challenge
[b] Data from Kligman 1966b; Number of subjects sensitized/total number tested.
[c] Kligman 1966b
[d] Pretreatment with 10% sodium lauryl sulfate

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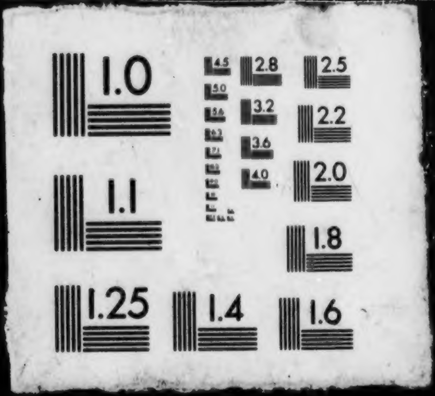
1.0
4.5
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1.1
1.8
1.25
1.4
1.6

Table 2-8. Reproducibility of Maximization Test Grades In Humans[a]

Substance	Induction Concentration %[d]	Sensitization Rates[b] Test No. 1	2	3	Grades[c] Test No. 1	2	3
Ammoniated mercury	25	13/25	11/24	15/25	III	III	IV
p-Aminobenzoic acid	25[d]	0/23	0/24	0/24	I	I	I
Apresoline[R]	5	25/25	24/24	25/25	V	V	V
Benzocaine[R]	25[d]	5/24	3/24	5/22	II	II	II
Chloroquine diphosphate	25[d]	9/24	6/25	11/23	III	II	III
Hexachlorophene	25	0/24	1/25	0/23	I	I	I
Monobenzyl ether of hydroquinone	25	22/22	25/25	24/25	V	V	V
Penicillin G	25[d]	16/23	12/23	13/25	IV	III	III
Tetrachloro-salicylanide	5	24/24	22/23	24/25	V	V	V
Tetramethyl-thiouram-disulfide (Thiran)	25	4/22	1/23	1/25	II	I	I

[a] Data taken from Kligman 1966c
[b] Number of sensitized subjects/total
[c] Grades: I - Sensitization rate; 0-2/25; weak
II - Sensitization rate; 3-7/25; mild
III - Sensitization rate; 8-13/25; moderate
IV - Sensitization rate; 14-20/25; strong
V - Sensitization rate; 21-25/25; extreme
[d] sodium lauryl sulfate provocative test

Table 2-9. Grades of Allergenic Potency By the Maximization Tests In Humans and Guinea Pigs

	Guinea Pig[a]				Human[b]			
Substance	Induction[c] Concentration (topical) %	Challenge[c] Concentration %	Positive %	Grade[d]	Induction[c] Concentration %	Challenge[c] Concentration %	Positive %	Grade[d]
Acrylic Monomer	5	10[e]	84	V				ND[f]
Aluminum Chloride	25	2	0	I	25	10	0	I
Apresoline[R]	5	1	80	IV	5.0[g]	5.0[h]	100	V
Atabrine[R]	25	10	90	V	25[g]	10[h]	78	IV
Benzocaine[R]	25	5	28	II	25[g]	10[h]	22	II
Formalin	5[e]	2[e]	80	IV	5	1.0	72	IV
Hexachlorophene	25	1.5	0	I	25[g]	10	0	I
Lanolin	25	15	0	I	25[g]	10[h]	0	I
Malathion[R]	10	20	54	III	25[g]	10[h]	100	V
Marfanil[R]	5	20	100	V				ND
Mercapto-benzothioazole	25	15	40	III	25[g]	10[h]	38	III
Mercuric Chloride	1.0	0.1[e]	32	III	2.0	0.05	92	V
Monobenzyl ether of hydroquinone	25	25	50	III	10	5	92	V
Neomycin	25[e]	25[e]	72	I	25[g]	10[h]	28	II
Nickel Sulfate	5[e]	0.5[e]	55	III	10[g]	2.5[h]	48	III
Penicillin G	5	10	100	V	25[g]	10[h]	67	IV
Potassium dichromate	1	0.1[e]	75	IV	2.0	0.25	100	V
Sodium lauryl sulfate	5	0.5	0	I	10	0.1	0	I
Streptomycin	10	0.5[e]	72	IV	25[g]	10[h]	80	IV
Sulfathiazole	25	10	36	III	25[g]	10[h]	4	I
Tetrachloro-salicylanilide	1	1[e]	72	IV	5.0[g]	0.5[h]	88	IV
Turpentine	25	20	64	III	50	20	72	IV
Tween 80	25	20	0	I	25[g]	5.0	0	I
Vioform[R]	25	5	20	II	25[g]	10[h]	0	I

[a] Results from Magnusson and Kligman 1969

[b] Results from Kligman 1966c,d

[c] Vehicle is petrolatum

[d] Maximization Grading from Kligman 1969
  Grade I - Sensitization rate, 0-8%; weak
  Grade II - Sensitization rate, 9-28%; mild
  Grade III - Sensitization rate, 29-64%; moderate
  Grade IV - Sensitization rate, 65-80%; strong
  Grade V - Sensitization rate, 81-100%; extreme

[e] Vehicle is water

[f] ND = not done

[g] Pretreatment of skin with sodium lauryl sulfate

[h] SLS provocative test

[i] Vehicle is 70% ethanol

Table 2-10. Sensitization Rate In Guinea Pig Closed Patch Test and Human Repeated Insult Patch Test[a]

	Human		Guinea Pig	
Substance (Vehicle)	Concentration[b] %	Sensitization %	Concentration[b] %	Sensitization %
Benzoyl peroxide/ 1.0% sulfur (PG)	10	40.5	10	42.1
Tetrachlorosalicylanilide (TCS) (1% ethanol)	0.05	19.0	1.0	80
Dithioquaternary Ammonium Cmpd (DA) aqueous surfactant	0.2	10.9	1.0 (1% ethanol)	100
Sulfonyl Compound	0.5	7.5	0.5	0
Hydroxylamine Sulfate	0.05 (aqueous detergent)	3.9	15 (aqueous detergent)	0
1-(3-Chlorophenyl)-3-phenyl-2-pyrazole (CPP)	1.0 (aqueous detergent)	2.9	0.05 (1% ethanol)	0
Ethanol	50	6.4	80	66.6
Neomycin (petrolatum)	5	1.5[c] (2.6)[d]	5	0
Benzocaine (petrolatum)	10	1.0[c] (3.0)[d]	10	0
Hexachlorophene (petrolatum)	0.5	1	10	0
Bithionol (petrolatum)	20	0[c] (0)[d]	20	0
Furacin (petrolatum)	0.2 1[c]	1[c] (3)[d]	0.15[e]	0

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[a] Data compiled from Griffith and Buehler 1977; Marzulli et al. 1968, Marzulli and Maibach 1974.

[b] Concentrations are for induction and challenge.

[c] Values without sodium lauryl sulfate.

[d] Values with Sodium lauryl sulfate.

[e] Induction (0.15%) and intradermal (0.15%) and topical (20%) routes for challenge.

7.0 USE OF HUMAN TESTS

Essentially, three types of tests (predictive, diagnostic and use) are required to evaluate skin sensitization potential. The Predictive test is needed to identify allergic substances. The procedures used in predictive tests include the Draize Test (Marzulli and Maibach, 1976b) or the Maximization Test (Kligman, 1966c). In the Research Institute for Fragrance Materials' Monographs on Fragrance ingredients, over 350 different ingredients were tested for sensitization potential in humans. Greater than 99.5% of the tests are now run using the Maximization procedure. The Diagnostic test is required to determine which substances are actually posing dermatologic problems and the Use test provides information on the safety of ingredients in a particular combination for a specific use. This information is partially derived from consumer complaints and injury reports.

Comparative diagnostic and predictive data in humans for 15 compounds are given in Table 2-11. In the Diagnostic test, wide geographic areas are tested with a standard screening kit to evaluate possible racial, ethnic, climatic and genetic variations in response. In this test, a preparation is applied to a clinical patient's skin under an occlusive patch for 48 hours. The skin is evaluated for evidence of erythema, edema or more severe skin changes occurring at 24, 48 and 72 hours after patch removal. Substances, concentrations and methos are standardized and the data are collected centrally, analyzed and evaluated in terms of a sensitization index (% of positive skin reactions). The differences in the response between the North American Contact Dermatitis Group (NACDG) and the International Contact Dermatitis Research Group (ICDRG) could be due to climatic or genetic differences or simply to the types of patients being tested (e.g., occupationally exposed, patients with intractable eczema, etc.). Consistently higher values were obtained in the Predictive test than in the diagnostic tests. This could have resulted from pretreatment with sodium lauryl sulfate, sodium lauryl sulfate provocative patches or high con- centrations of the chemicals used.

The results displayed in Table 2-11 permit the following judgements: (1) When a sensitization frequency in Diagnostic test exceeds that of the Predictive test (e.g., parabens), individuals are being identified who cannot tolerate a widely used product. (2) When the Diagnostic and Predictive test sensitivities show a high frequency, the substance is a strong sensitizer (e.g., p-phenylenediamine) and (3) when the Diagnostic and Predictive test frequencies are low, the Predictive test is accurately forecasting a low allergenic potential, not a false negative reaction.

The results of the Use tests are more difficult to evaluate since the products are generally confined to use at low concentrations and at varying frequencies. For example, formaldehyde (aqueous solution), which is recognized as a strong sensitizer and according to the Cosmetic Product Registry of the FDA (1972) is used in about 5% of 8,000 preparations, has failed to sensitize large numbers of the general population primarily because it is used in low concentrations as a preservative in shampoos and other products. Most of these preparations are rinsed off after use and do not remain in contact with the skin for long periods. On the other hand, the use of formaldehyde as a nail hardener is accompanied by significant injury to sensitive nails and adnexal tissues.

Table 2-11. Evaluation of Some Common Sensitizers By Diagnostic Test and Predictive Test

	Diagnostic Test[a]						Predictive Test[a]				
	NACDG[b]			ICDRG[b]			Concentration %				
Compound[c]	Concen-[d] tration %	Numbers of Reactors	%	Concen-[d] tration %	Numbers of Reactors	%	Induc- tion	Chall- enge	Total No.	Reactors %	Type[a] of Test
Nickel Sulfate	2.5	131	11[e]	5.0	321	6.7	10	2.5	25	48	MT
Potassium dichromate	0.5	91	8	5.0	318	6.6	2	0.25	23	100	MT
Thimerosal	0.1	91	8	--	--	--	--	--	--	--	
p-Phenylene-diamine	1.0	98	8[e]	1.0	237	4.7	0.2	2.5	24	29.1	MT
Ethylenediamine	1.0	85	7	--	--	--	1.0	1.0	88	53.4	DT
							5	1.0	61	8.0	DT
Neomycin Sulfate	20	71	6[e]	20	176	3.7	25	10	25	28[f]	MT
							20	20	42	16.6[f]	MT
							5	5	186	1.6	
Benzocaine	5	54	5	5	192	4.0	25	10	23	22[f]	MT
							10	10	173	1.2	DT
							20	10	99	6.0	DT
Ammoniated mercury	1.0	65	5	--	--	--	25	10	25	52[f]	MT
Mercaptobenzo-thiazole	2	58	5[e]	2	99	2.0	25	10	24	37.5[f]	MT
Formalin (aqueous)	2	43	4	2	169	3.5	5	1.0	25	72[f]	MT
							5	1.0	52	7.7	DT
							10	1.0	102	7.8	DT
Woolwax alcohol	30	37	3	30	127	2.6	--	--	--	--	
Thiram	2	50	4[e]	2	97	2.0	25	10	25	16[f]	MT
							1.0	1.0	309	0.3	DT
Parabens mixture[g]	15	38	3[e]	15	91	1.9	5.0M[f] +1.25P	same	98	1.0	DT
Dibucaine HCl	1.0	32	3	--	--	--	--	--	--	--	--
Cyclomethycaine Sulfate	1.0	23	2	--	--	--	--	--	--	--	--
Total Tested		1,200			4,824				1,419		

[a] Diagnostic test data taken from Marzulli and Maibach, 1974, 1976b; Predictive test data taken from Kligman, 1966c (maximization test; MT) and Marzulli and Maibach, 1973, 1974; (Draize test; DT)

[b] Data from the North American Contact Dermatitis Group (NACDG) - 1200 male and female, black and white subjects and from the International Contact Dermatitis Research Group (ICDRG) - 4824 male and female, white subjects.

[c] All compounds prepared in petrolatum, except formalin.

[d] Same concentration of test substance was used for induction and challenge.

[e] NACDG vs ICDRG, significant at 95% level.

[f] Pretreatment of skin with Sodium lauryl sulfate and Sodium lauryl sulfate provocative test.

[g] Methyl, ethyl and propyl parabens in equal amounts in the diagnostic test; methyl (M) and propyl (P) parabens in unequal amounts in the predictive test.

Two important considerations must be kept in mind when identifying potential human allergens. One is the size of the test group which should be small enough to be logistically feasible in the laboratory and large enough so that the results are valid for the general population; the other is the ability to predict the outcome of the test under conditions of use.

The complex mathematical problem of extrapolating from small test populations to the general population has been reviewed by Henderson and Riley (1945). Using the generally accepted 95% confidence limit, a finding of no skin reaction in a test population of 200 random subjects indicates that 22 of every 1,000 members of the general population may react and at the 99% confidence level, 15 of every 1,000 may react. If the test population is 100, up to 30 of every 1,000 members of the general population may react (95% confidence). Conversely, 1 (one) positive reactor out of every 200 subjects on test indicates that a population of 10,000 subjects contain from 1 to 275 sensitized individuals (95% confidence level). Thus, at least 30,000 subjects must be studied to be assured of no risk (i.e., 0.01% maximum permissible percentage of reaction in the population at 95% confidence limit). Obviously, this is a cumbersome task, while extrapolation is uncertain.

8.0 CONCLUSIONS

A review of the comparative data from various sources indicates that the guinea pig is the most widely used animal model for detecting skin sensitization potential. Except for the Draize test, all other procedures reviewed have some potential for detecting "weak" sensitizers. No one test method appears to be suitable for testing of all compounds, because significant variations in results have been observed from different sources using the same test method, identical compound, and same concentrations, (Table 2-2, e.g., Penicillin G, Maximization Test).

The Maximization Test using guinea pigs appears to be one of the better tests for detecting the weaker sensitizers that must be avoided in drugs, cosmetics and toiletries. Lesser agreement between guinea pigs and humans was noted for industrial chemicals and pesticides. The test virtually eliminates the false negative results seen with the Draize Test, but does not appear to be adequate for predicting sensitization to finished products. Application of this test to other chemical classes, such as industrial chemicals, drugs, and dyes, would require extensive validation in guinea pigs with follow-up testing in humans.

The Optimization, Open Epicutaneous and the Closed Patch Tests may be viable alternatives to maximization but the published evidence on their efficiency is sparse. All three of these tests, however, can be used on finished products. The Closed Patch Test involves a subjective evaluation of the sensitivity, but allows testing of the actual user concentration. The Open Epicutaneous Test is also flexible in this regard. Several of the commercial and industrial test labs contacted used both the Maximization and Closed Patch Test with success, but no statements were made as to which one is a superior method.

When the tests reveal that a chemical possesses immunologic capabilities, the percent of animals sensitized does not necessarily indicate the probable incidence of human sensitization. A negative result is predictive and indicative of relativly low hazard. More precise predictions of the potential for human sensitization will only be obtained by extensive clinical testing.

9.0 RECOMMENDATIONS

Identification of a preferred, single or pair of methods would require a thorough validation with industrial chemicals, pesticides and consumer products, including drugs and cosmetic and fragrance ingredients. Costs for the individual methods vary but the two most widely used differ by only approximately 15% making either method nearly equivalent on this basis.

When testing a compound for sensitization, the use of a known sensitizer as a positive control, such as dinitrochlorobenzene, is recommended to aid in scoring and personnel training. Proper handling is necessary to prevent inadvertent sensitization of laboratory staff. If positive controls are not used, then the guinea pig strain must be specified and the stock's continued sensitivity should be checked periodically, as it is possible to breed insentive animals that may be used in these tests.

Careful validation of the method chosen by the individual investigator is also strongly advised, because there may be class differences in a chosen method's specificity.

A satisfactory skin sensitization program for industrial chemicals, new drugs, pesticides, paints and coatings, toiletries, and cosmetics should involve testing in the guinea pig and if possible, in man. The substance should be tested first in the guinea pig to determine whether it is a low grade or potent sensitizer. Strong positive results with the guinea pig clearly indicate that the substance is not suitable for human use under uncontrolled conditions; moderately positive results, in many cases, would preclude the use of the substance in food and cosmetic products and general industrial exposure; a negative result could indicate a need for further characterization and testing.

The next step in a complete evaluation of sensitization or of weak and negative sensitizers might involve the use of provocative procedures (high concentrations, abrasions, use of sodium lauryl sulfate, etc.) in a small group of human subjects to determine if the individual compounds or formulated product is likely to sensitize at all. If results are negative, the use test should be done; if the results are positive, a standard human Patch Test without adjuvant (Draize Test) should be run on a suitable population. The combined findings will reveal whether a more expanded Use test is necessary.

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PHOTOTOXICITY

1.0 Summary

Phototoxicity and photoallergy testing are in an evolving status and well-defined proven tests are not available. Yet these important toxic properties should not be ignored when assessing the toxicity of photoactive compounds. The action spectrum for photosensitization is normally similar to the absorption spectrum of the photoactive compound. Methods for phototoxicity testing in animals and humans are summarized in Tables 3-3 and 3-4 and methods for photoallergy testing in guinea pigs are summarized in Table 3-5. Rabbits, hairless mice, guinea pigs and miniature swine are presently acceptable animal models for phototoxicity testing. The guinea pig is the only animal model presently acceptable for photoallergy testing. Since extrapolation of animal photosensitivity data to humans is often difficult, human testing is recommended for compounds to which humans will be exposed in the presence of sunlight. Human testing of a substance should be conducted only after testing in animals for both photosensitivity and systemic toxicity.

2.0 PHOTOSENSITIVITY

Photosensitivity has not generated a great deal of interest outside of the cosmetic field and no testing has been mandated by regulation. Guidelines on this subject are being prepared by the OECD.

The involvement of light should be considered whenever the toxicity of light-absorbing chemicals is being assessed. Photosensitivity refers to both photoallergic and phototoxic (nonimmunologic) light-induced skin responses. Phototoxicity can be considered the light-induced counterpart of primary irritation and photoallergy is the light-induced counterpart to contact dermatitis. Table 3-1 shows a summary of the similarities and differences between phototoxic and photoallergic mechanisms.

Selected studies in humans and guinea pigs indicate that many compounds can be both phototoxic and photoallergic. Most compounds which have been reported as photoallergic in humans can be shown to be phototoxic in guinea pigs provided the concentration is sufficiently high and the appropriate wavelength of exciting light is used (Harber and Shalita, 1977).

Chemicals with phototoxic and photoallergic potential have several chemical features in common. Most are dicyclic and tricyclic aromatic compounds that fluoresce e.g., phenanthrene (Harber and Shalita, 1977). The absorption spectrum of both phototoxic and photoallergic chemicals is usually in the ultraviolet (UV) range, but may extend into the visible region. Listed in Table 3-2 are examples of phototoxic and photoallergic agents.

The conditions prevailing in the skin at a particular site or time can influence the susceptibility of the skin to photoallergy (Harber and Shalita, 1977). These conditions include: 1) both the quality and location of the photosensitizer on the skin; 2) the capacity of the chemical to penetrate the skin through percutaneous absorption as well as trauma such as abrasion or sunburn; 3) the pH, the presence of enzymes and solubility conditions at the site of exposure; 4) the durations and intensity of exposure to activating radiation; 5) the depth of penetration of the activating radiation; 6) the humidity and ambient temperature; 7) thickness of the horny layer of skin; 8) the presence and degree of pigmentation; and 9) the immunologic state of the subject or laboratory animal. These above conditions may also affect phototoxicity with the exception of #9.

Table 3-1. Comparison of Phototoxic and Photoimmunologic Reaction[a]

Reaction	Phototoxic	Photoimmunologic
Reaction possible on first exposure	Yes	No
Incubation period necessary-first exposure	No	Yes
Chemical alteration of photosensitizer	No	Yes
Covalent binding with carrier	No	Yes
Clinical changes	Usually like sunburn	Varied morphology
Flares at distant previously involved sites possible	No	Yes
Persistent light reaction can develop	No	Yes
Cross-reactions to structurally related agents	Infrequent	Frequent
Broadening of cross-reactions following repeated photopatch testing	No	Possible
Concentration of drug necessary for reaction	High	Low
Incidence	Usually relatively high (theoretically 100%)	Usually vary (but theoretically could reach 100%)
Action spectrum	Usually similar to absorption	Usually higher wave length than absorption spectrum
Passive transfer	No	Possible
Lymphocyte stimulation test	No	Possible
Macrophage migration inhibition test	No	Possible

[a]Adopted from Harber and Baer, 1972.

Table 3-2. Phototoxic and Photoallergic Chemicals[a]

Phototoxic Compounds	Photoallergic Compounds
Acridine	Bithional
Anthracene	Chloropromazine
Chlorothiazides	Fentichlor
Coal Tar	Griseofulvin
Pesticides	Halogenated salicylanilides
Salithion	4-chloro-2-hydroxybenzoic acid N-m-butylamide
Cyanophos	
Dichlorvos	6-Methylcoumarin
Chlorothalonil	
Maneb	Promethazine
Paraquat	
Phenanthrene	
Psoralens	
Pyridine	
Sulfonamides	
Sulfonylureas	

[a] Adopted from Harber and Shalita, 1977 and Horiuchi et al., 1978

3.0 PHOTOTOXICITY TESTING

3.1 Animal Models

Testing for phototoxicity (light induced irritation) has not developed to the point that specific tests including animal models have become standardized. Under appropriate test conditions, several species can be utilized for phototoxicity testing (Marzulli and Maibach, 1970). The rabbit and the hairless mouse appeared to be more sensitive to 5-methoxypsoralen than the guinea pig. Miniature swine were less reactive but "stripping" of the skin with cellophane tape enchanced responsiveness; the squirrel monkey appeared resistant. The hamster showed histological changes due to phototoxicity; however, these were not apparent on gross examination. Forbes et al. (1977) have tested a number of fragrance raw materials using both sunlight and different laboratory light sources on the skin of hairless mice, humans and miniature swine. The hairless mouse skin was the most sensitive, the reactions in humans and swine were qualitatively and quantitatively similar.

Dose: Dosing should be on a microgram or milligram-per-square centimeter basis, simplifying the extrapolation to dosing in humans (Maibach and Marzulli, 1977). The chemical can be delivered to the skin with a micropipette. Following application, the animals are exposed to ultraviolet light from a high-output source. Most responses to phototoxic chemicals are elicited by high intensity, U.V. light above 310 nm. One high dose normally is administered, since no situations have been reported in which a compound has been negative at high dose and positive at lower doses. Each animal may be used as its own control. Control should include negative (the vehicle), positive (a known relevant phototoxic chemical such as methoxypsoralen), and a chemically treated site which is not irradiated. Experience suggest that most chemicals that are phototoxic by cutaneous exposure will produce toxicity in the majority of the animals; therefore, small groups of from 4 to 10 animals are sufficient for this testing.

Response: Phototoxic response is generally elicited quickly; therefore, for a positive effect, the site should be irradiated within 30 minutes to 2 hours after the chemical application. Examination and grading are performed 12 to 24 hours later.

Specific methods for phototoxicity testing in laboratory animals have been published by Marzulli and Maibach (1970), Alkin et al. (1979), and Forbes et al. (1977) and are summarized in Table 3-3. The three methods are similar but vary in specific details. One method uses intraperitoneal injection rather than skin application. The rabbit and hairless mouse are found to be sensitive species for phototoxicity screening studies. In one study the swine skin sensitivity compared favorably with human skin. For the chemicals tested, the hamster and monkey were poor animal models. Further test development must occur before a standarized screening test can be recommended.

3.2 Human Testing

Extrapolation of animal phototoxicity data to humans is difficult. In these cases, human testing may be necessary if the basic systemic toxicity data

Table 3-3. Phototoxicity Testing in Animals

Animal Model	Site Preparation and Application	Light Exposure	Observation and Scoring	Reference
Hairless Mice CF-1 Swiss Mice Hartley guinea pigs	Intrapertioneal Injection (25-50 mg/kg)	30 minutes after drug treatment; Blacklight 16 hrs. 2 cm above mice 10 cm above guinea pig	6 to 18, and 48 hours post-irradation Scoring 0-4	Akin *et al*. (1979)
Hairless mice Miniature swine	20 micro 1 test material on 2 cm^2 on back skin	30 minutes after application; a. sun (glass filtered) 30 min. b. compact-arc xenon lamp-2 minutes c. long-arc Xenon lamp-40 minutes d. black-light-60 min.	4,24,48,72 and 96 hours Scoring + or - (not graded)	Forbes *et al*. (1977)
Rabbits Hairless mice Mice Hamsters Squirrel monkeys Guinea pigs	Hair was removed by clipping and 0.05 ml of test material applied.	5 minutes after application Hanovia "Inspectolite" 90% UV (between 300 - 400 mm) 3000 microwatts/cm^2 at a distance of 10 cm. Irradiated 20-30 minutes.	24 and 48 hours Scoring + or - (not graded).	Marzulli and Maibach (1970)

are available. Human tests can be safely performed because only small test areas are exposed to compounds found relatively safe in animals. The greatest hazard to the experimental subject is the possible development of a small area of dermatitis, which should heal promptly. Before any test involving humans, fully informed consent of the subject must be obtained. The experimental procedure in humans resembles that used with animals; however, because human skin is less permeable than that of most laboratory animals it is usually necessary to make the skin more permeable by removing most of the stratum corneum with repeated cellophane tape stripping. The control site is stripped in the same manner. The dose should be administered to the test site in one small application.

Phototoxicity testing procedures in humans have been published by Marzulli and Maibach (1970), Kaidbey and Kligman (1978), and Forbes et al. (1977) and are summarized in Table 3-4. A major difference is the time between application and light exposure. The ideal time interval between application and light exposure hasn't been determined. Further research is required before specific tests can be recommended.

Table 3-4. Phototoxicity Testing in Humans

Site Preparation and Application	Light Exposure	Observation and Scoring	References
Topical application on white volunteers. (Details not given).	30 minutes after application: a. Sun (glass filtered)- 30 min. b. Compact-arc xenon lamp - 2 min. c. Long-arc xenon lamp - 40 min. d. Blacklight - 60 min.	4,24,48,72, & 96 hours Positive (+) or negative (-)	Forbes et al. (1977)
White volunteers (ages 21-28) - 50 1 of test material applied topically	6 hr. after application Xenon solar simulator, flux was 120 m w/can 2 for 8.5 min., if no reaction up 14 min. (28.5 Joules/cm^2).	Immediately after irradiation, 24 and 48 hours. Positive or negative (no grading).	Kaidbey and Kligman (1978)
Volunteers, skin of forearm was tapped stripped and 0.05 ml of test material applied.	5 minutes after application Hanovia "Inspectolite" 90% UV (between 300-400 nm) 300 watts/cm^2 at a distance of 10 cm. Irradiated 40 minutes.	24 and 48 hours Scoring + or - (no grading)	Marzulli and Maibach, (1970)

4.0 PHOTOALLERGY TESTING

4.1 Animal Model

In contrast to the several species used for phototoxicity testing, the guinea pig is considered to be the only reliable model for studying the immunologic reactions of photoallergy. Harber and Shalita (1977) described a method using guinea pigs and irradiating with either fluorescent "Sunlamp" tubes or "Blacklight" fluorescent tubes. The procedure consists of topical exposure and irradiation performed three times during a seven day period. Three weeks after the last sensitizing exposure, the animals are challenged by topical application to two sites on an area not previously exposed to the chemical. Thirty minutes later, one of the sites is exposed to non-erythrogenic radiation. All sites are scored and interpreted 24 hours later.

Other groups have published methods using the guinea pig model (Harber et al., 1967, and Vinson and Borelli, 1966). These two methods are summarized in Table 3-5 and are shown to be very similar; however, the rest period between the induction phase and challenge was 7-10 days versus 21 days. The ideal time for challenge has not been established.

4.2 Human Testing

Kaidbey and Kligman (1980) have developed a photomaximization test for identifying photoallergic contact sensitizers. This test consists of applying the test chemical to 25 subjects under occlusive patch for 24 hours followed by exposure to three minimal erythema doses (MED) of solar radiation. The irridiation consists of six exposures, twice weekly for 3 weeks. The subjects are challenged 10-14 days later. An occlusive application is made at a new site for 24 hours followed by exposure to 4.0 joules/cm^2 of UV A irradiation (lamp intensity, 27.0 nW/cm^2). Controls include a treated non-irradiated site and an irradiated vehicle-treated site. The reactions are evaluated 48 and 72 hours after irradiation.

Table 3-5. Photoallergy Testing in Guinea Pigs

Induction Phase	Challenge	Light Exposure	Observation and Scoring	Reference
Skin clipped and depilated. 0.1 ml test material applied 3 times during 7-day period.	3 weeks after induction 0.05 ml applied to previous unexposed area.	30 minutes after application. Sunlamp 1x10^7 ergs/cm^2 Blacklight 3x10^8 ergs/cm^2 Used successively. Blacklight only for challenge.	24 hours after irradiation. Scoring 0-4	Harber et al. 1967
Skin clipped on upper dorsal area and 0.05 ml test material applied daily for 5 days.	After rest period of 7-10 days 0.05 ml test agent applied.	Time after application not given. Sunlamp at distance of 18 in. for 15 minutes after each application. Same after challenge dose.	24 hours after irradiation. Scoring 0-3	Vinson and Borselli, 1966

5.0 CONCLUSIONS

Phototoxicity and photoallergy testing are in an evolving status and well-defined proven tests are not available. Yet these important toxic properties should not be ignored when assessing the toxicity of photoactive compounds. The action spectrum for photosensitization is normally similar to the absorption spectrum of the photoactive compound. Methods for phototoxicity testing in animals and humans are summarized in Tables 3-3 and 3-4 and methods for photoallergy testing in guinea pigs are summarized in Table 3-5. Rabbits, mice, hairless mice, guinea pigs and miniature swine are presently acceptable animal models for phototoxicity testing. The guinea pig is the only animal model presently acceptable for photoallergy testing. Since extrapolation of animal photosensitivity data to humans is often difficult, human testing is recommended for compounds to which humans will be exposed in the presence of sunlight. Human testing of a substance should be conducted only after testing in animals for both photosensitivity and systemic toxicity.

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SYSTEMIC DERMAL TOXICITY

1.0 SUMMARY

Dermal toxicity testing is performed to determine whether a substance can be absorbed in quantities sufficient to produce systemic effects, as well as the nature of such effects. Some of the factors that influence the degree of irritation produced by an agent will also influence its systemic toxicity. These include characteristics of test agents such as pH and lipid/water solubility and specific test procedures, such as abrasion of the skin and the methods used to apply test substances. A dermal study alone will rarely be sufficient to completely characterize the toxic effects of an agent, as it provides information on the effects produced by only one route of exposure. The OECD guidelines suggest testing the rat, rabbit or guinea pig. With the IRLG guidelines, preference is given to the rabbit. The data evaluated indicate that the rat is a more appropriate species to study systemic effects after dermal exposure. This is primarily because much of the available toxicity data resulting from tests by more conventional routes of exposure have been obtained from the rat. If LD50 values from different routes are compared in the rat, the relative rate of percutaneous absorption of a series of compounds can be estimated.

A comparison of LD50 values for rabbits and rats shows that, in more than 75% of the documented cases, the LD50's varied by less than a factor of four, with neither species clearly showing greater sensitivity. It has also been shown that the LD50 values were similar whether a rabbit was used for 24 hours or a rat was used for 4 hours.

Dermal toxicity studies of longer duration are limited in their practicality and cost-effectiveness. Animal restraint and patch attachment are necessary for long periods. The assessment of systemic effects may also be complicated by irritation or infection that can result from long term application of the test material. In addition, systemic effects can be adequately determined by administration of the agent by other routes which are cost-effective. Likewise, extensive clinical chemistry measurements and histopathology studies should be selectively performed, depending on the intended use of the substance. Inclusion of these additional measurements can be appropriate, however, in tests carried out by other routes.

When dermal toxicity studies are performed, particular attention should be paid to the size of the patch, the area of skin in relation to the size of the animal, the degree of occlusion, and the concentration and amount of test substances to allow development of consistent data.

2.0 INTRODUCTION

The Testing Guidelines of the Organization for Economic Cooperation and Development (OECD, 1981) for dermal toxicity include three tests that differ in terms of the duration of exposure, numbers of animals and extent of clinical chemical and histopathologic evaluation. These three tests are the Acute, Repeated Dose (21/28 day), and Subchronic (90 day) Dermal Toxicity Studies. The EPA originally proposed similar guidelines under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Toxic Substance Control Act (TSCA). Public comment was solicited on these guidelines and has been tabulated and examined by the EPA. No public comments were available for consideration from the OECD and Interagency Regulatory Liaison Group (IRLG, 1981), which also have prepared guidelines.

Although a great number of comments were received, many of the issues raised concerning the FIFRA and TSCA guidelines are being resolved by adopting the OECD guidelines. Several issues still remaining to be clarified include choice of species, exposure time, frequency of bleeding for hematology and clinical chemistry, washing of insoluble compounds from the skin following exposure, application of solid materials, use of abraded skin, application procedures, extent of histopathology and clinical chemistry requirements, difficulty in dose quantitations, and the circumstances or conditions that dictate the need for long-term dermal testing. Some issues covered by public comments are accounted for in the IRLG proposed test protocols but are not covered by the OECD. To facilitate discussion, the various test methods are compared for acute dermal LD50 in section 4.0 and subchronic dermal toxicity in section 5.0.

Little information is available on comparisons of dermal LD50's, methodologies, and interspecies variation in toxicity. Since the skin of the rabbit and guinea pig is usually more permeable to chemical substances than human skin, care must be taken when using dermal toxicity data in animals to predict potential adverse effects in man. In some respects, dermal toxicity studies are analogous to inhalation studies. In neither cases is a fixed amount of test substance administered to the test species as it is in an injection or an intubation study; but instead the animal is exposed to a concentration of the test substance. The actual dose received is a function of the amount of test substance absorbed through the skin or lung tissue. For inhalation, the concentration of the chemical in the chamber is determined; for dermal toxicity, the amount of material applied to a given surface area or the amount applied per unit of body weight is the usual expression of dose.

3.0 FACTORS INFLUENCING ABSORPTION AND TOXICITY

The extent to which a chemical is absorbed through the skin is determined by the physical and chemical properties of the test material and the structural characteristics of the skin at the site of application.

3.1 Properties of Test Agents

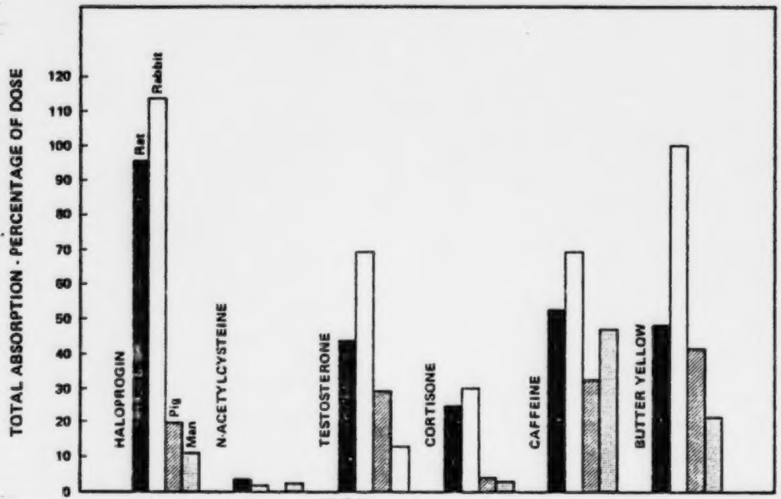
Physicochemical properties of the test agent have the greatest influence on whether it penetrates the stratum corneum of the epidermis which is the major barrier of the skin. Lipid soluble compounds are normally absorbed better than water soluble compounds. However, amphoteric compounds such as DMSO with both hydrophobic and hydrophilic groups usually demonstrate the greatest absorption. Other factors such as pH, pK (degree of ionization), temperature, humidity, and molecular size will also influence penetrability. Absorption can also take place through the follicular and sweat ducts. Inadvertent inhalation of a volatile test agent may contribute to the development of toxicity, compounding the difficulty of accurately determining the actual dose given via the dermal route (Casarett and Doull, 1980).

Although the skin is generally an effective barrier against the penetration of a variety of chemical agents, many substances do penetrate to some degree. Some of the better known examples of agents that can cause percutaneous toxicity are: organophosphate insecticides, chlorinated hydrocarbon insecticides, solvents, some aromatic nitro and amino compounds, organometallic compounds and steroids.

Because the stratum corneum acts as a barrier to passive diffusion, steady penetration rates are proportional to the concentration gradient across the membrane. Quantitative measurements of dermal toxicity are therefore dependent on the amount and concentration of the test agent, and the size of the test area in relationship to the total surface area or weight of the animal. For example, a given weight of test substance applied to 1% of the total surface area of an animal could be non-toxic, whereas, if it were applied to 10% of the surface, toxicity might result as total uptake would be proportional to surface area. The same dosage calculated on a mg/kg basis could give disparate results. These variables should be controlled rigidly so that valid conclusions and comparisons can be drawn from toxicity studies. As with dermal irritation studies, control of the patch test unit (patch size, material, tape, degree of occlusion.) is critical in keeping variability in dermal toxicity studies to a minimum.

3.2 Species Differences

Bartek et al. (1972) compared the percutaneous penetration of 6 test chemicals in rats, rabbits, miniature swine, and man by measuring the percent of radioactivity excreted in urine for 5 days after treatment with labeled compounds. The agents studied were haloprogin, N-acetylcysteine, cortisone, testosterone, caffeine, and butter yellow. The percent of the dermal dose (4 ug/cm^2) divided by the percent of an i.v. dose recovered in the urine was used to estimate the amount of test material absorbed through the skin. The data presented in Figure 4-1 indicate that skin permeability increased in the following order: man, miniature swine, rat, and rabbit. The swine, therefore, had permeability characteristics closest to man. With the exception of caffeine, there was a good correlation between the ether/water partition coefficients and percutaneous absorption, implying that this could be used to predict the absorption potential of a compound.



Roudabush et al. (1964) compared the acute dermal toxicity of 8 common chemicals in rabbits and guinea pigs. Three of these chemicals, acetone, benzene, and carbon tetrachloride were non-toxic by dermal application in either species, regardless of whether intact or abraded skin was used. Butylamine was more toxic on intact guinea pig abdomen than on either abraded guinea pig or rabbit back. Aniline, acrylonitrile, and parathion were more toxic in rabbits while butoxyethanol was more toxic in guinea pigs. These results are summarized in Table 4-1. The authors concluded that the differences between LD50 values for rabbits and guinea pigs (intact skin) were similar to what is seen when a chemical is tested by 2 laboratories in the same species. They added that there was no scientific basis for preferring the rabbit as a test animal. The observation of similar LD50's is not to be equated with equivalent percutaneous absorption of the compounds in different species. Variability in organ specificity, transport, absorption and metabolism from one species to another can greatly affect both percutaneous uptake and lethality.

Webster and Maibach (1975) investigated the percutaneous absorption of hydrocortisone, testosterone, and benzoic acid in the rhesus monkey and man. The total percentages absorbed for each agent were similar in monkey and man, suggesting to the authors that the rhesus monkey would be a good model for predicting results in humans. From a cost and availability standpoint however, the rhesus monkey is impractical for routine testing. In pigs, rats, and rabbits, testosterone was absorbed in much greater quantities than in the rhesus monkey and man.

Table 4-1. Acute Dermal LD50 (ml/kg) of Test Compounds in the Rabbit and Guinea Pig

Compound	Rabbit, abraded skin	Guinea Pig, (Abdomen) intact skin	Guinea Pig, (Back) abraded skin
Acetone	9.4	9.4	9.4
Benzene	9.4	9.4	9.4
Carbon tetrachloride	9.4	9.4	9.4
Butylamine	1.5	0.58	1.5
Aniline	0.82	1.29	2.15
Butoxyethanol	0.68	0.23	0.30
Acrylonitrile	0.28	0.46	0.84
Parathion	0.07	0.60	0.80

Adapted from Roudabush et al (1964)

In a number of cases the rabbit LD50 parallels the rat LD50 and in many instances they agree quite well. Weil et al. (1971) showed for 33 unspecified compounds that the rat LD50 after 4 hours correlated very well with the rabbit LD50 after 24 hours. When data from the Registry of Toxic Effects of Chemical Substances (RTECS) data base for 72 compounds is considered (Appendix) the rabbit dermal LD50 differs by a factor of two or less in 42 instances (greater than 65%) and differs by three or less in a total of 58 cases (greater than 80%) and overall by a factor of five or less in 63 cases (87%). Most recent price quotes for acute dermal toxicity determinations indicate that a rabbit test is 2.5 to 3 times more costly than a similar protocol in rats. In addition, the smaller size of the rat would allow testing of less material, an important consideration if the compound under test is part of a small pilot batch. These facts and the argument made by the National Academy of Sciences (1977) support the use of the rat over the rabbit. "Although there is always risk in extrapolation from animals to humans, it is usually safe to presume that substances with lower dermal LD50s in animals will be potentially more toxic to humans than those with higher LD50's. On the other hand, predictions of dermal versus oral toxicity in humans are more difficult, especially if the dermal and oral measurements are made in different animal species. Therefore, there is an important advantage in having both tests done with the same species, e.g., the rat."

3.2.1 Recommendations

In summary, the results of the preceeding investigations indicate that no one animal species will accurately predict the dermal effects of all classes of chemicals in humans. Although the guinea pig and rabbit are generally more sensitive than man, a reasonable approximation of expected toxicity can be obtained by use of either species. The rat also appears to be a valuable test animal for dermal studies because the availablity of toxicity data by other routes of exposure will provide information on the relative rate of percutaneous absorption. Use of monkeys is impractical for routine testing. The choice of species for dermal toxicity studies, therefore, should not be rigidly defined.

3.3 Regional Variation in Absorption

Because of functional and structural differences, the regional variation in the percutaneous absorption of parathion, malathion, and carbaryl was investigated by Maibach et al. (1971). Using humans, a test concentration of 4 ug/cm^2 was applied to the forearm, palm, abdomen, dorsum of the hand, scalp, angle of the jaw, postauricular area, forehead, intertriginous axilla, or the scrotum. Quantitation was based on the 5 day collection of urine with determination of radioactivity. These results, depicted in the following table, demonstrate that considerable variation in absorption rates exist for different chemical structures and regions of the body. Results are shown in Table 4-2.

Table 4-2. Effect of Anatomic Region on Absorption of Topically Applied ^{14}C Pesticides in Humans

	Percentage Dose Recovered in Urine		
Anatomic Region	Parathion	Malathion	Carbaryl
Forearm	8.6	6.8	73.9
Palm	11.8	5.8	--
Foot, ball	13.5	6.8	--
Abdomen	18.5	9.4	--
Hand, dorsum	21.0	12.5	--
Fossa cuubitalis	28.4	--	--
Scalp	32.2	--	--
Jaw, angle	34.0	--	69.9
Postauricular	34.0	--	--
Forehead	36.3	23.2	--
Ear canal	45.6	--	--
Axilla	64.0	28.7	--
Scrotum	101.6	--	--

Adapted from Maibach et al (1971)

3.4 *In Vitro* vs. *In Vivo* Absorption

Franz (1975) conducted an *in vitro* percutaneous absorption study with 12 chemicals and compared these results with those from previously conducted *in vivo* studies (Feldman and Maibach, 1970). The *in vitro* studies used human abdominal skin taken at autopsy; the *in vivo* experiments were conducted on the forearm of volunteers. Although quantitative agreement between the *in vivo* and *in vitro* studies was not good, there was significant (P less than 0.01) correlation of the *relative* permeability trends, salicylic acid and caffeine being the only exceptions. These results are summarized in Table 4-3. Franz concluded that the study of percutaneous absorption in excised human skin gives information which is relevant to its living counterpart. It is apparent however, that these large differences in quantitative rates should be taken into account when comparing interspecies data. The *in vitro* skin is in a much different environment, not being perfused by oxygenated blood on the basal side, and displaying differences in metabolism as absorbed compound/metabolites are not swept away into the bloodstream. Questions of effective skin thickness and epidermal metabolism as well as the temperature at which the test is performed make *in vitro* tests for compound absorption difficult to interpret.

Table 4-3. Total Absorption In Human Skin

Compound	In Vivo[a]	In Vitro[b]
1. Hippuric acid	0.2 ± 0.1 (7)	1.2 (0.8, 2.7) (15)
2. Nicotinic acid	0.3 ± 0.1 (3)	3.3 (0.7, 8.3) (19)
3. Thiourea	0.9 ± 0.2 (3)	3.4 (2.4, 5.5) (52)
4. Chloramphenicol	2.0 ± 2.5 (6)	2.9 (1.0, 5.7) (12)
5. Phenol	4.4 ± 2.4 (3)	10.9 (7.7, 26) (7)
6. Urea	6.0 ± 1.9 (4)	11.1 (5.2, 29) (22)
7. Nicotinamide	11.1 ± 6.2 (7)	28.8 (16, 65) (21)
8. Acetylsalicylic acid	21.8 ± 3.1 (3)	40.5 (17, 49) (14)
9. Salicylic acid	22.8 ± 13.2 (17)	12.0 (2.3, 23) (10)
10. Benzoic acid	42.6 ± 16.5 (6)	44.9 (29, 53) (18)
11. Caffeine	47.6 ± 21.0 (12)	9.0 (5.5, 20) (17)
12. Dinitrochlorobenzene	53.1 ± 12.4 (4)	27.5 (19, 33) (18)

[a] Percentage of applied dose, mean ± standard deviation. The figure in brackets is the number of subjects studies.

[b] Percentage of applied dose, median with 95% confidence interval given in parentheses.

Adapted from Franz (1975) and Feldman and Maibach (1970)

3.5 Use of Abrasion

Because abrasion removes or damages the stratum corneum, it is generally agreed that this technique will increase the toxicity of a chemical substance. This has been clearly shown by Frosch and Kligman (1977) to be valid for irritation in their development of the scarification index (the concentration of a substance necessary to produce irritation on normal versus abraded skin). An increase in percutaneous absorption of chemical agents after stripping the stratum corneum has also been demonstrated (Blank et al., 1975; Bettley, 1963).

An increase in absorption would produce a greater concentration of an agent at target organs and thus, increase its toxicity. If a chemical is likely to come in contact with damaged skin, the use of abraded skin in animal studies is justified. However, as this represents an unusual condition of the skin, the use of abrasion in routine testing should probably not be required.

3.6 DERMAL LD50 VS ORAL LD50

Gaines (1960) compared the acute oral and dermal LD50 values of a variety of chlorinated hydrocarbon, organophosphate and miscellaneous pesticides in the rat. He noted that, in general, female rats were more sensitive than males to organic phosphate compounds. There was a much closer relationship between dermal LD50's and the occurrence of occupational poisoning than between oral LD50's. These comparisons are shown in the following table.

Table 4-4. Relationship Between Toxicity and Safety of Pesticides

Compound	LD50 values in male rats (mg/kg)		Kind of exposure associated with systemic poisoning	Greatest severity of occupational poisoning
	Oral	Dermal		
DDT	11.3	2510	Ingestion	--
Lindane	88	1000	Ingestion	--
Dieldrin	46	90	Ingestion and occupational	Severe
Methyl parathion	18	65	Occupational	Mild
Guthion	13	220	Occupational	Mild
Parathion	13	21	Ingestion and occupational	Severe
Thimet	2	6	Occupational	Severe
Phosdrin	6	5	Occpational	Severe

Adapted from Gaines (1960)

A comparison of LD50 values by 2 different routes will give an approximation of the relative penetrability of chemicals through the skin. In the study by Weil et al. (1971), correlation between dermal LD50 and oral LD50 values of 30 substances was poor, indicating that the substances were absorbed at different rates.

Dermal and oral LD50 values for a number of chemicals in a variety of species are reported in the Registry of Toxic Effects of Chemicals (RTECS). These have been abstracted and are listed in the Appendix. Dermal LD50 values are greater or equal to the oral LD50 in over 84% of the cases. The greater toxicity via the oral route is also evident in many other mammals, and birds as well as rats, rabbits, and humans.

4.0 ACUTE DERMAL TOXICITY TESTING

The acute dermal toxicity test is performed with young adult animals. The use of rabbits, rats and guinea pigs is recommended by OECD while IRLG has chosen rabbits as the preferred species, because of ease of handling, size and skin permeability (Table 4-5). Other species may be used in either test but require justification. The FHSA protocol for acute dermal toxicity testing specifies that the intact and abraded skin of rabbits be used. Only the OECD and IRLG protocols allow the use of a limit test; if a dose of 2 g/kg or 2 ml/kg does not lead to mortality, no further testing of acute dermal toxicity may be necessary. OECD requires intact skin for the acute limit test while IRLG requires abraded skin. This may significantly increase the sensitivity of the IRLG protocol. The acute oral limit test is 5 g or 5 ml/kg. Dermal application is limited by the volume of material that can be applied uniformly and the available surface area. To apply greater than 2 g/kg body weight would in many cases involve repeated application or covering much greater than 10% of the animal's surface with compound. Interpretation and quantitation of the LD50 would therefore, be quite difficult. If a substance is toxic in the limit test, acute dermal toxicity testing will still be necessary. However, if toxicity is not demonstrated a considerable amount of cost and time will be saved.

4.1 Duration of Exposure

Weil et al. (1971) determined the dermal LD50 values of 30 unspecified substances in rats and rabbits after 4 and 24 hours of skin contact as well as for 33 additional substances using only the 4 hour rat and 24 hour rabbit exposure. The procedure used was a modification of the Draize method (1955). The LD50 value at the shorter time of exposure correlated very well with the longer time in both species. Moreover, there was a good correlation of the logarithmically adjusted LD50 values for the rat 4-hour and rabbit 24-hour exposure, indicating that either test method will provide adequate information to assess the toxicity of a substance.

The authors noted that the 4-hour skin test was more realistic in simulating expected exposures to man. The data obtained from the 4-hour rat study appears to be as useful as that from the 24-hour exposure of the rabbit in determining the relative toxicity to animals. This observation should be verified with various classes of compounds to establish its generality. This study does not address, for instance, other acute effects besides lethality. The information provided by these investigators, however, seems to be adequate justification for the use of a 4-hour exposure, and also indicates the usefulness of the rat as an experimental animal.

Table 4-5. Comparison of Guidelines for Acute Dermal Toxicity

	OECD	IRLG	EPA
Animals			
Species	Rabbit, rat or guinea pig; justify others	Albino rabbits preferred Justify others	Same as IRLG
Sex	Male and female	Same	Same
	Females should be nulliparous and not-pregnant	Females should not be pregnant	Same as IRLG
Age	Young adult	Same	Same
Weight	Rabbit - 2.0-3.0 kg Rat - 200-300 g Guinea Pig - 350-450 g	Rabbits - Same Not applicable Not applicable	Rabbits - Same Same as IRLG Same as IRLG
Acclimation	At least 5 days	Not mentioned	Same as IRLG
Study Design			
No./Group	5/sex	4/sex recommended	Same as IRLG
Limit Test	2 g/kg on intact skin of 3 males and 3 females	2 g/kg on abraded skin of 3 males and 3 females	5 g/kg on abraded skin of 4 males and 4 females
	If not mortality, further testing not necessary	Same	Same
Dose Levels	At least 3 levels spaced appropriately to produce test groups with a range of toxic effects and mortality rates. Permits LD50 determination and dose response curve.	Same	Same
Maximum Dose	Not mentioned	Maximum dose 2 g/kg	Not mentioned
Controls	Not required	Same	Same

Table 4-5. Comparison of Guidelines for Acute Dermal Toxicity

(Continued)

	OECD	IRLG	EPA
	Influence of vehicle on skin penetration should be considered	Vehicle should be non-irritating and of known low toxicity. Consider effect on absorption	Acute test of vehicle first if toxic potential is not known. Should be non-toxic and not modify toxic response of test substance
Duration of Exposure	24 hours. Then removal of residual with water or other appropriate solvent	Same	Same
Condition of test substance	Liquids applied undiluted	Liquids - Same	Liquids - Same
	Solids pulverized and moistened with water or other vehicle	Solids - As paste with water or saline	Solids - Same as IRLG
Condition of test area	Intact skin	Same	Same
	Not less than 10% of body surface	Same	Same
	Apply to dorsal surface	Same	Apply in a band around the trunk
Preparation	Clip fur shortly before test	Clip fur 24 hours prior to testing	Same as IRLG
	If shaving or chemical depilation used, must be 24 hours prior to test	No other methods mentioned	Same as IRLG
Retainer	Porous gauze dressing or nonirritating tape	Same	Wrapping material (type not specified)
	Dressing should be covered	Cover with impermeable material in a semi-occlusive fashion	See above

Table 4-5. Comparison of Guidelines for Acute Dermal Toxicity

(Continued)

	OECD	IRLG	EPA
Study Conduct			
Husbandry	Conditions of temperature, humidity, lighting, feeding, and caging specified	Reference to DHEW (NIH) 74-23	Similar to IRLG
Observation	Frequently first day, twice a day thereafter, at least 4 hrs apart (morning and late afternoon)	Same	Same
Duration	At least 14 days	Same	Same
	Record onset, severity and persistence of signs	Same	Same, plus nature of sign
	Irritation not graded	Irritation noted and graded at 24 and 72 hours	Irritation noted and graded - no time specified
Evaluation			
	Weigh initially, weekly and at death	Same	Same
Necropsy	Consider for all animals where significant signs of toxicity are observed	All animals that die during test. Terminate animals if no further testing planned or if results are to be used for labeling purposes	Same
Histopathology	Consider for organs with evidence of gross pathology (in animals surviving 24 hr or more)	Not mentioned	Not mentioned
Reporting of Results	General requirement sufficient to make independent evaluation	Detailed format specified	Same as IRLG
		Requirements designated in GLP's	Same as IRLG

5.0 LONGER TERM DERMAL TOXICITY TESTING

The requirement to test an agent for longer periods should be based on the type of exposure expected in man. Substances such as sun screen lotions or insect repellents that will be applied to a large portion of the body for extended periods of time should undergo longer term dermal toxicity studies. Agents that will only be in contact with the skin rarely and for a brief time probably need not be tested as rigorously.

Pre-chronic studies are often useful in setting doses for chronic study, but the data generated must also be justified in terms of time and cost. Once the dermal LD50 of a substance is determined, the long term effects of an agent might be better established by conducting a subchronic or chronic toxicity study by another route of exposure because such studies will probably still be required. Systemic effects after dermal exposure also may not occur due to insufficient percutaneous absorption. Limit tests on intact skin are therefore included for subchronic dermal testing allowing the possibility of testing only one high dose group for the full exposure period if no effects are observed at 1 g/kg.

Three different tests have been included in OECD guidelines: a 21 or 28 day repeated dose, a 90 day subchronic, and a chronic test (Table 4-6). For subchronic dermal toxicity studies the OECD, IRLG and EPA all recommend that ideally, exposures should be performed 7 days/week. This may not be practical because the test animal will require clipping of hair approximately once a week and therefore there will be no proper period for healing of any associated and often imperceptible clipping injury before the next week's dosing schedule is begun. Daily exposures will also require increased expenditures and staffing by the laboratory for weekend dosing. Testing during the weekend may not provide enough additional toxicological information to justify the added expense. For these reasons, OECD's guideline that dosing be performed 5 days/week with clipping performed on a non-dosing day seems most reasonable. An exposure schedule of 5 days per week also simulates many industrial exposures. Although this procedure might allow recovery over the weekend, this effect will be minimized if maximum tolerated doses are used. The weekend free of exposure may also, however, permit the loss of tolerance built up during the week.

A comparison of acute and subchronic dermal toxicity methods shows significant procedural differences. All of the guidelines for acute testing specify a 24 hour exposure period. For subchronic testing, exposure is for six hours a day, five to six days a week; the duration of exposure is 21, 28 and/or 90 days depending on which guidelines are followed. For acute studies, observations are made frequently the first day and twice daily, thereafter, for a total of at least 14 days. Observations are made daily throughout the period of exposure for subchronic testing. At evaluation for acute testing, the IRLG and EPA specify necropsy of all animals that die during the test but assessment of histopathological changes is not required. According to the OECD, necropsy should be considered where significant signs of toxicity are observed and histopathological studies should be considered for organs with evidence of gross pathology in animals surviving 24 hours or more. For subchronic toxicity testing, all of the guidelines specify that all animals should be necropsied, and histological evaluation be performed on all animals in the high dose and control groups. The guidelines for subchronic dermal toxicity testing differ from those for acute testing by specifying that extensive clinical tests be performed in some cases. These include hematology, blood chemistry, and ophthalmological measurements.

Table 4-6. Comparison of Guidelines for Subchronic Dermal Toxicity

Subject	OECD	IRLG	EPA
Species	Rat, Rabbit or Guinea Pig	Albino Rabbit or Rat	Albino Rabbit (preferred); others accepted if justified scientifically
Sex	Both sexes Non-pregnant & nulliparous	Same	Same as IRLG
Age/weight	Rabbit: 2-3 kg Rat: 200-300 g G.P.: 350-450 g	Same (Rabbit) Same (Rat)	Same (Rabbit)
No. of Animals	10M/10F per dose	At least 5M/5F Rabbits or 10M/10F Rats	At least 5M/5F per dose when preliminary tests (i.e. oral LD50, dermal LD50 or subchronic oral) indicate no sex differences in response to test substance. If sex difference are noted or such information is lacking, at least 8M/8F per dose
Dose Levels	At least 3 plus extra high dose group on 90 day study (sentinnel)	At least 3 highest dose non-irritating but effective	At least 3
Control	Control or vehicle control	Same	Same
Limit Test	If toxic effects produced at 1 g/kg or higher, no further dermal required	Maximum quantity of a test substance plus vehicle to be applied should not exceed 2 g/kg. Use abraded and non abraded sites	10M/10F to a single exaggerated dose which is maximum feasible that does not casue severe local irritation.

Table 4-6. Comparison of Guidelines for Subchronic Dermal Toxicity

(Continued)

Subject	OECD	IRLG	EPA
			Need not exceed 2 g/kg. If severe dermal irritant at level of human or anticipated human exposures, it need not be subjected to a 90-Day Subchronic Dermal test.
Preparation of Animals	Clip fur	Same - skin may be weekly	Same as OECD
Application of test substance	Apply as thin and uniform film.	Not specified	Same as OECD
Area	Uniformly over at least 10% of the body surface	Same, however Decreased area allowed	Same as OECD
	Liquid substances generally not diluted	Use same volume of application for all test doses	Dilution allowable if compound used in diluted form
	Not stated	Same	Calculate concentrations per unit area of skin
Duration	Ideally 7 days/ week at least 6 hrs/day for 21/28 days or 90 days, 5 day/week acceptable	Same as OECD, exposure for 90 days only - washing permitted	Same as IRLG plus 21 day test
Type of Covering	Porous gauze dressing; non-irritating tape; prevent animal from ingesting test substances	Semi-occlusive with impermeable material (i.e. plastic or rubberized cloth)	Same as OECD

Table 4-6. Comparison of Guidelines for Subchronic Dermal Toxicity

(Continued)

Subject	OECD	IRLG	EPA
Observations	Daily	Same	Same
	Body weight and food consumption weekly	Body weight weekly	Body weight weekly (if affected, food consumption weekly)
	No observation of irritation	Note Irritation weekly	Note for 21 days
Immobilization	No	No	No
Housing	Individual caging	Same	Same
Clinical Laboratory Tests			
Hematology and Blood Chemistry	Before study starts and at termination	Rabbits before study begins; rabbits and rats at end of study	Before and at end
	Hematocrit, hemoglobin, total/differential leucocyte count, platelet count, erythrocyte count	Same as OECD plus plus intrinsic and extrinsic clotting potential	Sames as OECD plus sed. rate and RBC count
	All animals at end of exposure	Same Rabbits before, at 6 weeks and at end of study. Rats at end of exposure only	High dose and control (initally) Same as OECD
	Calcium, inorganic phosphorous, chloride, potasssium, sodium magnesium, blood pH, BUN, fasting glucose, albumin, bilirubin. Consider lipids, hormones,	Same plus alkaline phosphatase, lactic dehydrogenase, direct and indirect bilirubin, gamma glutamyl transpeptidase ornithine decarboxylase. Same as OECD plus carboxyhemoglobin. No methemoglobin	Same plus blood CO2, magnesium, sorbitol dehydrogenase, glucose 6 phosphatase, isocitrate dehydrogenase, ornithine transcarbamylase, lactic dehydrogenase (plus isozymes) -

Table 4-6. Comparison of Guidelines for Subchronic Dermal Toxicity

(Continued)

Subject	OECD	IRLG	EPA
	and base balance, methemoglobin, cholinesterase for 21/28 day test Same as IRLG for 90 day test		cholinesterase if organophosphate or carbamate at beginning, twice during study and at end in red blood cell. Examine brain cholinesterase at termination. Same as IRLG
Ophthalmo logical	90 day test only	At least at start and end of test	Not required
Gross Necropsy	All animals	Same	Same
Organs weighed	Liver, kidney, adrenal, testes	Same as OECD	Same as OECD
Histopathology	All animals in high dose and control.	Same	Same
	21/28 days: Examine treated and untreated skin liver, kidney, plus target organs Examine lower doses in target organ 90 days:	Same as OECD requires for 21/28 day study plus thyroid, spleen, testes/ovaries, bone marrow, representative lymph node, salivary gland, small intestine, urinary bladder, thymus adrenal, lung, gall bladder plus	Same as OECD requires for 21/28 days
		Brain (3 levels); spinal cord (2 levels); sciatic nerve; pituitary; thyroid/parathyroid, trachea, esophagus, stomach, pancreas, aorta, prostate,	Routine exam of brain and spinal cord only if indicated by clinical signs or CNS effects

Table 4-6. Comparison of Guidelines for Subchronic Dermal Toxicity

(Continued)

Subject	OECD	IRLG	EPA
		uterus, heart, large intestine, sternum	
	Same as IRLG plus accessary genital organs, duodenum, jejunum, ileum, caecum, colon, rectum, and 3 levels of the spinal cord		

6.0 EXAMINATION

6.1 Irritation Assessment

In both the dermal LD50 and subchronic dermal toxicity, IRLG includes the grading of skin irritation as part of the observation for clinical signs 30 minutes following removal of the test patch and again at 72 hours after compound application. The grading system is similar to the Draize (1955) scoring systems but also includes noting the occurrence of severe eschar formation and/or corrosion (see Dermal Irritation). The OECD guidelines do not include recording of irritation. Many public comments made in response to the FIFRA and TSCA Test Guidelines recommend that the acute dermal toxicity and irritation tests be combined. The consideration of this recommendation should be given high priority, because if the acute test is properly designed, it can provide information relevant to the irritation potential. This would, of course, only apply when the rabbit is used to determine acute dermal toxicity.

Inclusion of irritation assessment in the dermal LD50 protocol would require further description of the patch test unit. Currently, no information on the patch size, concentration of test substances or conditions of the skin is given in any of the proposed guidelines. Without this information, some caution must be exercised in drawing conclusions on the relative toxicity and irritation of the various test agents.

6.2 Histopathology and Clinical Measurements

Both the OECD and IRLG suggest a "battery" of clinical chemical parameters and tissues for histopathological evaluations. Extensive monitoring of clinical chemistry and hematological parameters has, historically, been associated with the need to assess human clinical health status. Recently, the trend has been to include a battery of clinical biochemistry tests (applicable for humans) in subchronic and chronic animal studies. These tests are being proposed regardless of the purpose of the study, test chemical, economic impact, or significance and usefulness of the data obtained. Although these tests are an indirect assessment of cellular status, proponents assert that they do reflect the function of living cells. Histopathology is seen as an evaluation of the structure of dead and artifically preserved cells. However, most of the available literature indicates that histopathology is more useful than clinical chemistry in evaluating the toxic effects of chemical substances.

6.2.1 Clinical Chemistry (derived in part from Public Comments on EPA Guidelines)

Numerous studies have been published illustrating the variability of clinical chemistry determinations and the ultimate reliance on histopathological examination to identify treatment-related responses (Buttar et al., 1976; Chow et al., 1977; Verschuuren et al., 1976; and Oser et al., 1975). The existence of a correlation between serum enzyme levels and a histopathological effect has long been a subject of discussion. A summary of relevant statements received by EPA are presented below.

Korsrud et al. (1972) found that microscopic examination was more sensitive than enzyme analysis. According to the latter authors: "The sensitivity and the degree of quantitation of responses varied between methods. Minimal histologic changes were seen at a low CCl_4 dose (0.0125 ml/kg). Alterations in the other criteria were not observed at this dose" (emphasis added). The sensitivity of several serum enzymes for the detection of induced liver damage in rats was subsequently reported by Korsrud et al. (1973). The authors concluded that histological examination of the liver was useful for assessing the sensitivity of serum parameters since histological evidence of damage was detectable at lower doses of each toxicant than were serum changes.

An inhalation study on peroxyacetyl nitrate in rats (Kruysse et al., 1977) revealed that the 4.1 ppm level produced histopathological changes in the respiratory tract without any significant adverse effects on the hematological parameters monitored.

Dose-dependent effects of coumarin or butylated hydroxytoluene were examined by Nievel et al. (1976) with the following conclusion: "It appears that studies on any individual, randomly selected enzymes and biochemical parameters are insensitive and possibly unsuitable indicators of toxicity unless an overall change in the pattern of blood chemical pathology is correlated with the early molecular changes underlying nucleic acid and protein synthesis that are detectable within hours of the administration of many compounds of relatively low toxic potential." They continued, "Our evaluation of the biochemical parameters widely used in the assessment of and monitoring toxic damage of the liver in the course of many safety assessment programes therefore indicated no reliable correlation between these analytical chemical changes and toxicity."

However, although the use of isoenzyme determination might be useful in noninvasive measurement of organ damage in a more specific fashion, histopathology once again was more sensitive. Cornish et al. (1971) reported that "although the use of isozyme patterns,...have become relatively common as an aid in clinical diagnosis, the technique has not as yet been widely used in toxicology". These investigators reported the markedly different pattern seen in response to liver and kidney damage in rats, but cautioned that preparation of isozyme assays required that "conditions of blood collection and treatment of samples be uniform and carefully controlled". The sensitivity of isozyme patterns compared with the degree of morphological damage to the liver or kidney of rats was reported by Grice et al. (1971). Generally, advanced degenerative changes, including necrosis, had occurred in both these tissues before enzyme alterations were seen.

By the time that function tests show a significant variation from the control range, a very large amount of histologically visible damage has usually occurred. Our experience on the Carcinogenicity Testing Program within the National Toxicology Program also suggests that extensive clinical chemistry measurements do not add to the information provided by histopathological determinations. Several special protocols for halogenated and aromatic compounds failed to demonstrate sufficient sensivity. Histopathology, on the other hand, detected lesions at 1/2 to 1/4 the dosage necessary to demonstrate liver and kidney tissue damage by clinical chemistry measurements.

Clinical chemistry and histopathology studies should be considered complementary. At times one is more sensitive and in other instances the other is more sensitive. Data from one may also give clues as to what to look for in the other type of investigation. Histopathology documents lasting or irreversible changes quite well, but transiet effects may be shown better by clinical chemistry if the right sample is taken at the right time.

In summary it seems evident that an extensive battery of clinical chemistry measurements should not be required for all substances. However, some agents that exert their toxicity by inhibition of an enzyme, cholinesterase inhibitors for example, should be monitored by appropriate techniques. The evidence suggests that histopathology can serve as the principal method for the detection of tissue damage with clinical chemistry measurements being utilized when appropriate.

6.2.2 Hematology

The effectiveness of the normal routine hematological evaluations in detecting slight toxicological cellular alterations apparently is also limited in comparison to histopathological examination. Van Logten et al. (1974) reported that no striking effects on hematological or biochemical parameters were seen, except for a doubling of the percentage of neutrophil granulocytes in the highest dosage group in rats fed sodium bromide for 90 days. However, histopathologically, a dose-related disturbance of the endocrine system and some of its target organs was found. The results of a chronic study on tetrasul in rats was published by Verschuuren et al. (1973) in which the hematological parameters were generally comparable to control, whereas microscopic alterations were observed in the liver and thyroid. Furthermore, in short-term comparative studies on tetrasul in six animal species, Verschuuren et al. (1973) found that histopathological changes in the liver and the induction of microsomal liver enzyme activity were the most sensitive parameters. No statistically significant dose-related changes in the hematological parameters were observed.

Histological examination was also shown to be a better indicator of toxicity than hematological determinations when hamsters, rats and rabbits were exposed to acrolein (Feron et al. 1978). The investigators found that: "Hematological values in rats and rabbits were not affected by acrolein, but in hamsters, females of the top dose group showed statistically significant increases in the number of erythrocytes, packed cell volume, hemoglobin content, and lymphocytes accompanied by a decrease in the number of neutrophilic leucocytes. All enzyme activities were within normal ranges and no statistically significant differences occurred between the various test groups and the control group" (emphasis added). It was further reported that marked histopathological changes were seen in different portions of the respiratory tract of all animals at the top dose. Hematological measurements, however, may detect changes occuring in the bone marrow and spleen.

6.2.3 Recommendations

In humans and veterinary clinical cases, histopathology is not always possible and clinical chemistry and hematology studies may be effective diagnostic tools. However, in animal toxicity studies, there is little support for requiring extensive clinical determinations as a screening procedure for possible toxic manifestations. The bulk of the data indicates that histopathology is the best method for the detection of adverse effects for most chemicals. In certain specific instances, however, clinical chemistry measurements and hematology studies will be the only means of monitoring toxicity.

7.0 ADDITIONAL PUBLIC COMMENTS

The following additional topics appeared in the public comments on proposed EPA guidelines for dermal toxicity studies and deserve consideration.

- The requirement for measurement of food consumption is generally more applicable for oral toxicity tests where the diet contains the test substance. In a dermal study, if weekly weighings indicate normal growth, there should be no need to measure food consumption.
- Many comments emphasized that preliminary studies might provide information that should lessen the requirements for acute and subchronic dermal studies. No demonstrable toxicity in a limit test (maximum dose) should eliminate the need for extensive testing at lower doses. This can either be due to a lack of absorption or a lack of systemic toxicity. Detoxification by the skin is also a possibility but is less likely. Likewise if no significant histopathologic changes occur at the highest dose, the requirements for further analysis at lower doses should be dropped.
- Several comments were made that rigorous requirements for dermal studies were unnecessary, costly, and provided little new information if studies by more conventional routes of administration had been previously conducted. Specific reference was made to target organ damage and information derived from clinical chemistry and histopathology.
- Other commenters felt that the "shot-gun" approach for clinical laboratory tests was not supported by the scientific literature and experience. They felt that there was no scientific basis for the specification of 14-16 clinical measurements.
- Pre-test bleeding was criticized as providing no useful information and being traumatic. Untreated or vehicle treated groups with large numbers of animals satisfy the requirement for control data.

8.0 CONCLUSION

Because of differences in permeability, and other variables such as the area of the patch and the amount and concentration of the test substance, it is extremely difficult to quantitatively predict the dermal effects of a chemical in man even after extensive animal studies. The same quality of information could be obtained by determining the relative rate of percutaneous absorption of a substance and integrating this information with toxicity studies by other routes of administration.

Sufficient scientific evidence has been assessed that indicates that no single species will accurately predict the dermal effects of all classes of chemicals in humans. The guinea pig and rat have been evaluated as possible alternatives to the rabbit with favorable results. A definite advantage in using the rat in dermal toxicity study is that much more information by other routes of exposure is available and costs are lower. A drawback is the difficulty in restraining rats and maintaining the patch for the desired length of the test. Skin permeability for both species is usually greater than in man, thus allowing a conservative extrapolation. A comparison of LD50 values indicates that for most chemical substances there is good agreement between the 2 species.

All of the variables that influence the reproducibility and accuracy of irritation studies are involved and further complicated in dermal toxicity studies. An area of critical concern is the patch test unit: The patch size, material, type of tape, and degree of occlusion can influence the results of a toxicity study. Standardization of the method of applying the patch, degree of occlusion, and the amount and concentration of test substance is essential if meaningful interpretations are to made.

The concentration of the test substance and the area of application in relation to the total surface area or body weight of the animal can also influence the outcome of a study. If the animals are all within a close range of body weights, a standard size patch applied to the same surface area will eliminate some problems. If the animals vary in size this must be taken into account when deciding on the test area and the size of the patch to be used. Whenever possible, the same concentration of test substance for the various dose levels should be used.

The use of abraded skin in dermal toxicity studies does not seem to be justified unless the agent is expected to come in contact with damaged skin. Non-uniformity of abrasive techniques and difficulty in interpreting results should rule out this procedure for routine testing.

Since some test substances are not removed simply by washing the skin with water, various suggestions on how to remove the agent without causing injury to the skin have been made. If exposure in humans is expected to be continuous because of the difficulty in removing materials such as glues or dyes, animal experiments should be designed to simulate this condition (i.e., the excess compound should be removed if possible by washing, but residues left on skin should serve as a model for actual exposure).

The frequency of blood sampling and the extent of hematological and clinical chemistry testing should be selective rather than requiring that every blood parameter be determined. Unnecessary bleeding of animals can be detrimental and could theoretically alter the outcome of the test. Cardy and Warner (1979) reported a severe weight gain depression when rats were regularly bled. Also, the value and sensitivity of hematological examination has been questioned. Evidence has been presented that suggests that histopathological examination will demonstrate tissue damage at lower doses before it is detectable by clinical studies. This is not to say that hematological testing has no value, but rather that flexibility in the guidelines is necessary.

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APPENDIX

Comparison of Oral and Dermal Lethality[a]

Chemical	Species	Oral LD_{50} (mg/kg)	Dermal LD_{50} mg/kg	Dermal/Oral	Rabbit/Rat	
Acetamide, 2-chloro-N,N-diallyl-	Rat	700	360	0.51		
Acetamide, 2-fluoro-	Rat	5.8	80	13.7		
Acetamide, 2-fluoro-N-methyl-N-(1-naphthyl)-	Rat	115	213	1.85		
	Mouse	200	372	1.86		
	Guinea pig	2	5	2.50		
Acetanilide, 2-chloro-N-isopropyl-	Rabbit	710	380	0.35		
Acetic acid, 2-(tert-butyl)-4,6-dinitro-m-tolyl ester	Rat	42	1,300	30.9		
Acetic acid, (2,4-dichlorophenoxy)-	Rat	370	1,500	4.05	Dermal	0.93
	Rabbit	--	1,400	--		
Acetic acid, diphenyl-, 3-fluoroethyl ester	Rat	6	4	0.67	Oral	0.10
	Rabbit	0.6	7	11.7	Dermal	1.75
Acetic acid, fluoro-, methyl ester	Rabbit	0.5	2	4.00		
Acetic acid, mercaptophenyl-, ethyl ester, S-ester with O,O-dimethyl phosphorodithioate	Mouse	150	2,620	17.5		
Acetic acid, thiocyanato-, isobornyl ester	Rabbit	630	6,000	9.52		
Acetone	Rabbit	5,300	20,000	3.77	Oral	4.24
	Rat	1,250	1,800	0.14	Dermal	11.11
Acrolein	Rabbit	7	52	7.43		
Acrylanilide, 3',4'-dichloro-2-methyl	Rat	1,800	1,780	0.99		
Acrylonitrile	Rat	82	148	1.81	Oral	1.13
	Rabbit	93	250	2.69	Dermal	1.69
	Guinea Pig	50	460	9.20		

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[a] Data obtained from Registry of Toxic Effects of Chemical Substances (RTECS), U.S. Department of Health and Human Services, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health. Cincinnati Ohio.

APPENDIX (Continued)
Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD_{50} (mg/kg)	Dermal LD_{50} mg/kg	Dermal/Oral	Rabbit/Rat
Acetophenone, 2',3',4',5',6'-pentachloro-	Rabbit	93	250	2.69	
	Guinea pig	50	460	9.20	
Allodan	Rat	940	1,000	1.06	
Ammonium, alkyldimethylbenzyl-, chloride	Rat	280	1,560	5.57	
Ammonium, alkyldimethyl(ethylbenzyl)-, chloride	Rat	300	1,420	4.73	
Ammonium, (2-chloroethyl)trimethyl-, chloride	Rabbit	150	232	1.55	
Aniline	Rat	250	1,400	5.60	
Aniline, p-chloro	Rat	370	3,200	8.65	
Aniline, 4,4'-(imidocarbonyl)bis(N,N-dimethyl-, hydrochloride	Mouse	480	300	0.63	
p-Anisidine	Rat	1,400	3,200	2.29	
Arsine, Dichloro (2chloro vinyl)-	Rat	50	24	0.48	
	Rabbit	--	6	--	
Calcium arsenate	Rat	20	2,400	120	
Sodium arsenite	Rat	70	150	2.14	
Potassium arsenite	Rat	14	150	10.7	
Benzaldehyde, p-nitro-	Rat	4,700	16,000	3.40	
Benzaldehyde, p-nitro-, oxime	Rat	180	7,100	39.4	
Benzamide, 2,6-dichlorothio-	Rat	757	1,000	1.32	
Benzene nitro-	Rat	640	2,100	3.28	
Benzene thiol-	Rat	46	300	6.52	
Benzhydrol, 4,4'-Dichloro-alpha (Trichloromethyl)	Rat	575	100	0.17	Oral 3.15
	Rabbit	1810	1870	1.03	Dermal 18.70

APPENDIX (Continued)
Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD_{50} (mg/kg)	Dermal LD_{50} mg/kg	Dermal/Oral	Rabbit/Rat
2-Benzimidazolecarbamic acid, methyl ester	Rat	6,400	2,000	0.31	
1-Benzimidazolecarboxylic acid, 5,6-dichloro-2-(Trifluoromethyl)-	Rat	283	700	2.47	
Benzimidazole, 2-(2-furyl)-phenyl ester	Rat	1,100	1,000	0.91	
Benzoic acid, benzyl ester	Rat	1,700	4,000	2.35	Dermal 1.00
	Rabbit	--	4,000	--	
Benzoic acid, p-hydroxy-, isopropyl ester, ester with isopropyl ethyl-phosphoramidothioate	Rat	28	188	6.71	Dermal 0.86
	Rabbit	--	162	--	
1H-2,1,3-Benzothiadiazin-4(3H)-one-2,2-dioxide, 3-isopropyl-	Rat	1,100	2,500	2.27	
Benzyl alcohol	Rabbit	1,040	2,000	1.92	
4,4'-Bipyridinium, 1,1'-dimethyl-, dichloride	Rabbit	--	236	--	Dermal 2.95
	Rat	57	80	1.40	
	Duck	1,200	1,260	1.05	
1-Butanol, 2-ethyl	Rabbit	1,200	1,260	1.05	
t-Butyl hydroperoxide	Rat	406	790	1.95	
Butyric acid, 4-(4-chloro-o-tolyl)oxy)-, sodium salt	Rat	700	1,000	1.43	
Butyric acid, 4-(2,4-dichlorophenoxy)-	Rat	700	800	1.14	
Butyric acid, ester with dimethyl-(2,2,2-trichloro-1-hydroxyethyl) phosphonate	Rat	1,100	7,000	6.36	
Carbamic acid, diethylthio-, S-(p-chlorobenzyl)ester	Rat	1,903	2,900	1.52	
Carbamic acid, (3-(dimethylamino) propyl)- propyl ester, hydrochloride	Rat	10,900	5,000	.50	

APPENDIX (Continued)
Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD_{50} (mg/kg)	Dermal LD_{50} mg/kg	Dermal/Oral	Rabbit/Rat
Carbamic acid, dimethyl-, 1-((dimethylamino)carbonyl)-5-methyl-1H-pyrazol-3-yl ester	Rat	25	600	24.0	
Carbamic acid, dimethyl-, 1-isopropyl-3-methylpyrazol-5-yl ester	Rat	11	5.6	0.51	
Carbamic acid, (mercaptoacetyl)methyl-, ethyl ester, S-ester with O.O-diethylphosphorodithioate	Rat	36	380	10.6	
Carbamic acid, methyl-, o-sec-butylphenyl ester	Mouse	173	340	1.97	
Carbamic acid, methyl-, o-cumenyl ester	Mouse	150	7,600	50.7	
Carbamic acid, methyl-, m-cym-5-yl ester	Rat	74	450	6.08	
Carbamic acid, methyl-, 2,3-dihydro-2,2-dimethyl-7-benzofuranyl ester	Human	11	10,000	909	Dermal 7.38
	Rat	5.3	120	22.6	
	Bird	0.42	100	238	
	Rabbit	--	885	--	
Carbamic acid, methyl-, 4-dimethylamino-m-tolyl ester	Rat	30	275	9.17	
Carbamic acid, methyl-, 4-dimethylamino-3,5-xylyl ester	Rat	14	1,500	107	
Carbamic acid, methyl-, O-(((2,4-dimethyl-1,3-dithiolan-2-yl)methylene)amino) deriv.	Rat	1	300	300	
Carbamic acid, methyl-, 2,3-(dimethylmethylenedioxy)phenyl ester	Rat	179	1,000	5.59	
Carbamic acid, methyl-, o-(1,3-dioxolan-2-yl)phenyl ester	Mouse	61	1,660	27.2	
	Rat	60	32.000	533	
Carbamic acid, N-methyldithio-, sodium salt	Rabbit	320	800	2.50	
Carbamic acid, methyl-, 3-(ethylthiomethyl)phenyl ester	Rat	411	1,000	2.43	

APPENDIX (Continued)
Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD_{50} (mg/kg)	Dermal LD_{50} mg/kg	Dermal/Oral	Rabbit/Rat	
Carbamic acid, methyl-, o-isopropoxyphenyl ester	Rat	83	800	9.64	Dermal	1.81
	Rabbit	--	1,450	-		
Carbamic acid, methyl-, m-(1-methylbutyl)phenyl ester mixed with carbamic acid, methyl-, m-(1-ethylpropyl)phenyl ester (4:1)	Rat	87	242	2.78	Dermal	1.65
	Rabbit	—	400	--		
Carbamic acid, N-methyl-, 4-(methylthio)-3,5-xylyl ester	Rat	60	350	5.83		
	Bird (Wild)	5	100	20.0		
Carbamic acid, methyl-, 1-naphthyl ester	Rabbit	710	2.000	2.82	Oral	2.84
	Rat	250	4.000	16.0	Dermal	0.50
Carbamic acid, methyl-, m-tolyl ester	Rat	268	6,000	22.4		
Carbanilic acid, m-chloro-, 4-chloro-2-butynyl ester	Rabbit	600	23,000	38.3		
Carbonic acid, dithio-, cyclic-5,5-(6-methyl-2,3-quinoxalinediyl) ester	Rat	1,100	500	0.46		
Carbonochloridic acid, methyl ester	Mouse	67	1,750	26.2		
Carbon tetrachloride	Rat	2,800	5,070	1.81		
Coumarin, 3-(alpha-acetonylbenzyl)-4-hydroxy-	Rat	1,550	1,400	0.90		
Coumarin, 3-chloro-7-hydroxy-4-methyl-, O-ester with O,O-diethyl phosphorothioate	Rat	16	860	53.8		
	Bird (Wild)	3	7.5	2.50		
m-Cresol	Rat	242	620	2.56	Dermal	3.31
	Rabb	--	2050	--		
o-Cresol	Rat	121	1,100		Dermal	0.81
	Rabbit	--	890	--		
p-Cresol	Rat	207	750		Dermal	0.40
	Rabbit	—	301	--		

APPENDIX (Continued)

Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD_{50} (mg/kg)	Dermal LD_{50} mg/kg	Dermal/Oral	Rabbit/Rat
o-Cresol, 4,6-dinitro-	Rat	10	200	20.0	
o-Cresol, 4,6-ditnitro-, sodium salt	Rat	26	200	7.69	
Crotonic Acid, 3-Hydroxy-, alpha-Methylbenzyl Ester, Dimethyl Phosphate (E)-	Rat	74	202	2.73	
Crotonic Acid, 3-Hydroxy-, Methyl Ester, Dimethyl Phosphate, (E)-	Rat	3.7	4.7	1.27	
	Mouse	4	40	10.0	
	Duck	4.63	11	2.38	
Crontonic Acid, 3-Methyl-,2-sec-Butyl-4,6-Dinitrophenol Ester	Rat	58	720	12.4	Dermal 1.04
	Rabbit	--	750	--	
Crontonic Acid, 2-(1-Methylheptyl)-4,6-Dinitrophenyl Ester	Rabbit	2,000	9,400	4.70	
Cyanamide, Calcium Salt (1:1)	Rabbit	1,400	590	0.42	
1,3,5-Cycloheptatriene	Rat	57	442	7.75	
Cyclohexane, 1,2,3,4,5,6-Hexachloro-	Rat	100	900	9.00	Oral 0.76
	Rabbit	76	500	6.58	Dermal 0.56
Cyclohexane, 1,2,3,4,5,6-Hexachloro-, gamma-isomer	Rat	76	500	6.58	
Cyclopropanecarboxanilides, 3',4'-dichloro-	Rabbit	3,028	3,038	1.00	
Decaborane (14)	Rat	64	740	11.6	Dermal 0.10
	Rabbit	--	71	--	
1,4:5,8-Dimethanonphthalene, 1,2,3,4,10,10-Hexachloro-6,7-Epoxy-1 4,4a,5,6,7,8,8a-Octahydro-, endo, endo-	Rat	3	12	4.00	Oral 2.33
	Rabbit	7	60	8.57	Dermal 5.00
1,4:5,8-Dimethanonphthalene, 1,2,3,4,10,10-Hexachloro-6,7-Epoxy-1,4,4a,5,6,7,8,8a-Octahydro-, endo, endo-	Rat	46	10	0.22	Oral 0.98
	Rabbit	45	250	5.56	Dermal 25.00

APPENDIX (Continued)
Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD_{50} (mg/kg)	Dermal LD_{50} mg/kg	Dermal/Oral	Rabbit/Rat
1,4:5,8-Dimethanonphthalene, 1,2,3,4, 10,10-Hexachloro-1,4,4a,5,8,8a-Hexahydro-, endo, endo	Rat	7	23	3.29	
1,4:5,8-Dimethanonphthalene, 1,2,3,4, 10,10-Hexachloro-1,4,4a,5,8,8a-Hexahydro-, endo, endo	Rat	39	98	2.51	
p-Dioxane	Rabbit	2,000	7,600	3.80	
m-Dioxane, 5-ethyl-5-nitro-2-propyl-	Rat	2,000	9,400	4.70	
Distannoxane, Hexabutyl	Rat	87	11,700	134	Dermal 0.08
	Rabbit	--	900	--	
Distannoxance, Hexakis(beta, beta-Dimethylphenethyl)-	Rat	2,630	1,000	0.38	
Disulfide, Bis(Dimethylphosphinothioyl) mixed with Disulfide, Bis (Diisopropylphospinothioyl) (75%:25%)	Rat	265	480	1.81	
Ethane, 2,2,-Bis(p-methoxyphenyol)-1, 1-1-Trichloro-mixed with O,O-Diethyl O-(2-Isopropyl-4-Methyl-6-Pyrimidinyl) Phosphorothioate	Rabbit	--	8,000	--	Dermal 1.00
	Rat	2,000	8,000	4.00	
Ethane, 1,2,-dibromo-	Rat	108	300	2.78	Oral 0.51
	Rabbit	55	300	5.45	Dermal 1.00
Ethane, 1,1,1-Trichloro-2,2-Bis (p-Chlorophenyl)-	Rat	113	1,931	17.1	Oral 2.21
	Rabbit	250	300	1.20	Dermal 0.16
	Guinea Pig	150	1,000	6.67	
	Rabbit	320	490	1.53	
Ethanol, 2-amino-	Rat	2,100	1,500	0.71	Oral 0.48
	Rabbit	1,000	1,000	1.53	Dermal 0.67
Ethanol, 2-butoxy-	Guinea Pig	1,200	230	0.192	
Ethanol, 2-(2-Butoxyethoxy)-, Acetate	Rabbit	2,600	1,500	0.58	
Ethanol, 2-Chloro-	Rat	91	84	0.92	Dermal 0.67
	Rabbit	--	56	--	

APPENDIX (Continued)
Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD_{50} (mg/kg)	Dermal LD_{50} mg/kg	Dermal/Oral	Rabbit/Rat
Ethanol, 2-ethoxy-	Rabbit	3,100	3,500	1.13	
Ethanol, 2-(2-ethoxyethoxy)	Rat	6,500	6,000	0.92	Oral 0.50
	Rabbit	3,620	16,400	4.53	Dermal 2.73
Ethanol, 2-methoxy-	Rabbit	890	1,280	1.44	
Ether, 2.4-Dichlorophenyl p-Nitrophenyl	Rat	740	5,000	6.76	
Ether, 2'-Hydrooxy-2,4,4'-Trichlorodiphenyl	Rat	3,700	9,300	2.51	
Formamide, N,N-dimethyl	Rat	2,800	5,000	1.79	Dermal 1.00
	Rabbit	--	5,000	---	
Formamidine, N'-(4-Chloro-o-Tolyl)-N,N-Dimethyl-	Rat	170	4,000	23.5	Oral 3.68
	Mouse	160	225	1.41	Dermal 0.16
	Rabbit	625	640	1.02	
Formamidine, N'-(4-chloro-o-tolyl)-N,N-dimethyl-, hydrochloride	Rat	225	4,000	17.8	
Formic acid, chloro-, ethylene ester	Mouse	1,100	2,000	1.82	
Formic acid, chloro-, isopropyl ester	Mouse	178	12	0.07	
	Rabbit	--	11,300	---	
Formic acid, chloro-, oxydiethylene ester	Mouse	813	2,000	2.46	
Formic acid, chloro-, propyl ester	Mouse	650	10	0.02	
Glyoxylonitrile, phenyl-, oxime, O,O-diethyl phosphorothioate	Rat	1,845	1,000	0.54	
Heptanethiol, methyl	Rat	85	1,954	23.0	
2,4-Hexadien-1-ol	Rat	2,140	1,010	0.47	
1,3-Hexanediol, 2-ethyl-	Rabbit	2,600	2,000	0.77	
Hydracrylic acid, phenethyl ester	Rat	7,800	10,000	1.28	

APPENDIX (Continued)
Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD$_{50}$ (mg/kg)	Dermal LD$_{50}$ mg/kg	Dermal/Oral	Rabbit/Rat
Imidocarbonic acid, (diethoxyphosphinothioyl)dithio-, cyclic ethylene ester	Bird (Wild)	1.8	10	5.56	
1,3-Indandione, 2-((p-chlorophenyl)-phenylacetyl)-	Rabbit	50	200	4.00	
Iron, carbonyl	Rabbit	12	240	20.0	
Isopropylamine	Rabbit	3,200	550	0.17	
Lactonitrile, 2-methyl-	Guinea pig	9	150	16.7	
	Rabbit	13.5	17	1.26	
Methane, isothiocyanato-	Rat	97	2,780	28.7	Dermal 0.01
	Mouse	97	1,820	18.7	
	Rabbit	--	33	--	
4,7-Methanoindan, 1,2,4,5,6,7,8,8-octachloro-3a,4,7,7a-tetrahydro-	Rabbit	100	780	7.8 7.8	
4,7-Methanoindene, 1,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-	Rat	40	119	2.98	
4,7-Methanoisobenzofuran, 1,3,4,5,6,7,8,8-octachloro-1,3,3a,4,7,7a-hexahydro	Rat	6.2	5	0.81	Oral 0.65
	Rabbit	4	12	3.00	Dermal 2.4
	Guinea pig	2	2	1.00	
1,3,4-Metheno-2H-cyclobuta(cd) pentalen-2-one, 1,1a,3,3a,4,5,5,5a,5b,6-decachloroctahydro-	Rabbit	65	345	5.31	
Naphthalimide, N-hydroxy-, diethyl phosphate	Rat	70	140	2.00	
Nicotine	Rat	50	140	2.80	Dermal 0.36
	Rabbit	--	50	--	
Nicotine, sulfate (2:1)	Rat	55	285	5.18	
2-Norbornanone, endo-3-chloro-endo-6-cyano-, O-(methylcarbamoyl)oxime	Rat	19	303	15.9	

APPENDIX (Continued)
Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD$_{50}$ (mg/kg)	Dermal LD$_{50}$ mg/kg	Dermal/Oral	Rabbit/Rat
5-Norbornene-2,3-dicarboximide, N-(2 ethylhexyl)-	Rat	2,800	470	0.17	
5-Norbornene-2,3-dimethanol, 1,4,5, 6,7,7-hexachloro-, cyclic sulfite	Rat	18	74	4.11	Dermal 2.26
	Rabbit	--	167	--	
Optunal	Bird (Wild)	0.75	1.3	1.73	
7-Oxabicyclo(2,2,1)heptane-2,3-dicarboxylic acid, disodium salt	Rat	51	750	14.7	Dermal 0.13
	Rabbit	--	100	--	
1,2,4-Oxadiazolidine-3,5-dione, 2-(3,4-dichlorophenyl)-4-methyl-	Rat	10,200	1,350	0.13	
1,4-Oxathiin-3-carboxamide, 5,6-dihydro-2-methyl-N-phenyl-	Rat	430	1,050	2.44	
2,4-Pentanediol, 2-methyl-	Rabbit	3,200	13,200	4.13	
3-Penten-2-one, 4-methyl-	Rabbit	1,000	5,150	5.15	
Phenethyl alcohol	Guinea pig	400	5,000	12.5	
Phenol	Rat	414	669	1.62	Dermal 1.27
	Rabbit	--	850	--	
Phenol, 2-sec-butyl-4,6-dinitro-	Rat	25	80	3.20	
Phenol, 4,4'-Isopropylidenedi-	Rabbit	2,230	3,000	1.35	
Phenol, pentachloro-	Rat	50	105	2.10	
Phenol, m-phenoxy-	Rat	1,211	2,750	2.27	
Phenol, 2,4,6-tris (dimethylaminomethyl)-	Rat	1,200	1,280	1.07	
Phosphine oxide, tris(1-aziridinyl)-	Rat	37	87	2.35	
	Mouse	292	375	1.29	
Phosphine oxide, tris(1-(1-methyl) aziridinyl)-	Rat	136	183	1.35	

APPENDIX (Continued)
Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD$_{50}$ (mg/kg)	Dermal LD$_{50}$ mg/kg	Dermal/Oral	Rabbit/Rat
Phosphonamidothioic Acid, Ethyl-, 4-(Methylthio)-m-Tolyl Ester	Bird (Wild)	3.2	75	23.5	
Phosphonic Acid, 1-(Butylamino) Cyclohexyl-, Dibutyl Ester	Rat	3,000	1,200	0.40	Dermal 0.42
	Rabbit	—	500	-	
Phosphonic Acid, (2,2,2-Trichloro-1-Hydroxyethyl)-, Dimethyl Ester	Rat	450	2,000	4.44	Oral 3.22
	Rabbit	1,450	5,000	3.45	Dermal 2.50
Phosphonium, (5-Chloro-2-Thienyl) Methyltributyl-, Chloride	Rat	455	1,000	2.20	
Phosphonodithioic Acid, Chloromethyl-, S,S-Diethyl Ester	Rat	35	79	2.26	
Phosphonodithioic Acid, Ethyl-, O-Ethyl S-Phenyl Ester	Rat	3	147	49.0	
Phosphonodithioic Acid, Methyl-, S-((N-Methoxycarbonyl)-N-Methylcarbamoyl)Methyl O-Methyl Ester	Rat	57	720	12.6	
Phosphonothioic Acid, Ethyl-, O-Ethyl O-(2,4,5-Trichlorophenyl) Ester	Rat	15	64	4.27	
Phosphonothioic Acid, Phenyl-, O-(4 Bromo-2,5-Dichlorophenyl) O-Methyl Ester	Rabbit	124	800	6.45	
Phosphonothioic Acid, Phenyl-, O-Ethyl O-(p-Nitrophenyl) Ester	Rat	8	25	3.13	
	Duck	3	400	333	
Phosphoramidic Acid, Isopropyl-, 4-(Methylthio)-m-Tolyl Ethyl Ester	Duck	1.68	24	14.3	
	Rat	8	72	9.00	
Phosphoramidic Acid, Methyl-, 4-tert-Butyl-2-Chlorophenyl Methyl Ester	Rabbit	400	2,000	5.00	
Phosphoramidocyanidic Acid, Dimethyl-, Ethyl Ester	Rabbit	16	35	2.19	

APPENDIX (Continued)
Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD_{50} (mg/kg)	Dermal LD_{50} mg/kg	Dermal/Oral	Rabbit/Rat
Phosphoramidothioic Acid, Acetimidoyl-, O,O-Bis(p-Chlorophenyl) Ester	Rat	3.70	25	6.76	
Phosphoramidothioic Acid, O,S-Dimethyl Ester	Rat	7.5	50	6.67	Oral 1.33
	Rabbit	10	118	11.8	Dermal 2.36
Phosphoramidothioic Acid, N-Ethyl-, O-(1-Isopropoxycarbonyl-1-Propen-2-YL) O-Methyl Ester	Rat	100	1,500	15.0	
Phosphoric Acid, (7-Chloro-Bicyclo (3.2.0)Hepta-2,6-Dien-6-YL)Dimethyl Ester	Rat	96	2,925	30.5	
Phosphoric Acid, 2-Chloro-1-(2,4-Dichlorophenyl)Vinyl Diethyl Ester	Rat	20	30	1 50	Oral 25.0
	Rabbit	500	400	0.80	Dermal 13.3
Phosphoric Aicd, 2-Chlorovinyl Diethyl Ester	Rabbit	3	18	6.00	
Phosphoric Acid, 1,2-Dibromo-2,2-Dichloroethyl Dimethyl Ester	Rat	250	800	3.20	
Phosphoric Acid, 3,2-Dichlorovinyl Dimethyl Ester	Rat	32	75	2.34	Oral 0.04
	Rabbit	1.25	107	85.6	Dermal 1.43
Phosphoric Acid, 2,2-Dichlorovinyl Methyl Ester, Calcium Salt mixed with 2,2-Dichlorovinyl Phosphoric Acid Calcium Salt	Mouse	250	3,040	12.2	
Phosphoric Acid, Diethyl Ester, Ester with 6-Chloro-3-(Hydroxymethyl)-2-Benzoxazolinone	Rat	35	400	11.4	
Phosphoric Acid, Dimethyl Ester, ester with 2-Chloro-N,N-Diethyl-3-Hydroxycrotonamide	Rat	17	125	7.35	Dermal 2.14
	Duck	3.81	26	6.82	
	Rabbit	--	267	--	

APPENDIX (Continued)
Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD_{50} (mg/kg)	Dermal LD_{50} mg/kg	Dermal/Oral	Rabbit/Rat
Phosphoric Acid, Dimethy Ester, ester with (E)-3-Hydroxy-N,N-Dimethylcrotonamide	Rat	16	42	2.63	Dermal 4.00
	Bird (Wild)	2	1.3	0.65	
	Rabbit	--	168	--	
Phosphoric Acid, Dimethyl Ester, ester with (E)-3-Hydroxy-N-Methoxy-N-Methylcrotonamide	Rabbit	11	107	9.73	
Phosphoric Acid, Dimethyl Ester, ester with (E)-3-Hydroxy-N-Methylcrotonamide	Rat	21	112	5.33	
	Duck	3.36	30	8.93	
	Bird (Wild)	1.6	4.2	2.63	
Phosphorodithioic Acid, S-(2-Chloro-1-(1,3-Dioxo-2H-Isoindol-2H-Isoindol-2-YL)Ethyl)	Rabbit	35	145	4.14	
Phosphorodithioic Acid, 5-((6-Chloro-2-Oxo-3(2H)-Benzoxazolyl)Methyl)0,0-Diethyl Ester	Rabbit	120	390	3.25	
Phosphorodithioic Acid, S(((p-Chlorophenyl)Thio)Methyl) 0,0-Diethyl Ester	Rabbit	1,250	1,270	1.02	
Phosphorodithioic Acid, S(((p-Chlorophenyl)Thio)Methyl) 0,0-Dimethyl Ester	Rat	98	190	1.94	
Phosphorodithioic Acid, S(((p-Dichlorophenyl)Thio)Methyl) 0,0-Diethyl Ester	Rat	61	652	10.7	
Phosphorodithioic Acid, 0,0-Diethyl Ester, S-Ester with N-Isopropyl-2-Mercaptoacetamide	Rat	8	100	12.5	Oral 1.06
	Rabbit	8.5	14	1.65	Dermal 0 14
Phosphorodithioic Acid, 0,0-Diethyl Ester, S-Ester with 3-(Mercaptomethyl)-	Rat	9	250	2.78	
Phosphorodithioic Acid, 0,0-Diethyl, S-((Ethylsulfinyl)Ethyl) Ester	Rat	3.5	192	54.5	
	Mouse	12	263	21.9	

APPENDIX (Continued)
Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD_{50} (mg/kg)	Dermal LD_{50} mg/kg	Dermal/Oral	Rabbit/Rat
Phosphorodithioic Acid, 0,0-Diethyl S-(2-Ethylthio)Ethyl) Ester	Rat	2	6	3.00	
Phosphorodithioic Acid, 0,0-Diethyl, S-(Ethylthio)Methyl Ester	Rat	1.10	2.50	2.27	
	Guinea Pig	20	20	1.00	
	Duck	2.55	203	79.6	
Phosphorodithioic Acid, 0,0-Diisopropyl Ester, S-Ester with N-(2-Mercaptoethyl)	Rat	770	3,950	5.13	
Phosphorodithioic Acid, 0,0-Dimethyl S-(2-Acetamidoethyl) Ester	Mouse	342	472	1.38	
Phosphorodithioic Acid, 0,0-Dimethyl Ester, S-Ester with N-Ethyl-2-Mercaptoacetamide	Rat	125	2,000	16.0	
Phosphorodithioic Acid, 0.0-Dimethyl Ester, S-Ester with N-Formyl-2-Mercapto-N-Methyl-	Rat	250	353	1.41	
Phosphorodithioic Acid, 0,0-Dimethyl Ester, S-Ester with 4-(Mercaptoacetyl) Morpholine	Rat	190	283	1.49	
Phosphorodithioic Acid, 0,0-Dimethyl Ester, S-Ester with 2-Mercapto-N-(2-Methoxyethyl)	Rat	600	1,600	2.67	
Phosphorodithioic Acid, 0,0-Dimethyl Ester, S-Ester with 2-Mercapto-N-Methylacetamide	Rat	152	353	2.32	
	Guinea Pig	350	1,000	2.86	
Phosphorodithioic Acid, 0,0-Dimethyl Ester, S-Ester with 3-(Mercaptomethyl)-1,2,3-Benzotriazin-4(3H)-One	Rat	13	220	16.9	
Phosphorodithioic Acid, 0,0 Dimethyl Ester, S-Ester with 4-(Mercaptomethyl)-2-Methoxy-delta(sup 2)-1,3,4-Thiadiazolin-5-One	Rat	20	25	1.25	Oral 3.15
	Rabbit	63	375	5.95	Dermal 15.0

APPENDIX (Continued)
Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD$_{50}$ (mg/kg)	Dermal LD$_{50}$ mg/kg	Dermal/Oral	Rabbit/Rat	
Phosphorodithioic Acid, 0,0-Dimethyl Ester, S-Ester with N-(Mercaptomethyl) Phthalimide	Rat	147	1,550	10.5	Dermal	2.04
	Rabbit	--	3,160	-		
Phosphorodithioic Acid, S,S'-p-Dioxane-2,3-Diyl 0,0,0',0'-Tetraethyl Ester	Rat	63	20	0.32	Dermal	4.25
	Rabbit	-	85	--		
Phosphorodithioic Acid, 0-Ethyl, S,S-Dipropyl Ester	Rat	34	60	1.77	Dermal	0.43
	Duck	1.26	11	8.73		
	Rabbit	--	26	-		
Phosphorodithioic Acid, S,S-Methylene 0.0.0'0'-Tetraethyl Ester	Rat	13	62	4.77	Dermal	14.35
	Guinea Pig	40	915	22.9		
	Rabbit	--	890	--		
Phosphorofluoridic Acid, Bis(1-Methylethyl) Ester	Mouse	37	72	1.95		
Phosphorothioic Acid, S-Benzyl 0.0-Diisopropyl Ester	Mouse	1,760	5,000	2.84		
Phosphorothioic Acid 0-14-Bromo-2-Chlorophenyl)-0-Ethyl-S-Propyl Ester	Rat	400	300	0.75	Oral	1.75
	Rabbit	700	472	0.67	Dermal	1.57
Phosphorothioic Acid, 0-(4-Bromo-2,5-Dichlorophenyl) 0,0-Diethyl Ester	Rat	52	1,000	19.2	Dermal	1.37
	Rabbit	-	1,366	--		
Phosphorothioic Acid, 0-(4-Bromo-2,5-Dichlorophenyl) 0,0-Dimethyl Ester	Rabbit	720	2,181	3.03		
Phosphorothioic Acid, 0-(2-Chloro-1-Isopropylimidazol-4-YL) 0,0-Diethyl Ester	Rat	40	290	7.25		
Phosphorothioic Acid, o-(2-Chloro-4-Nitrophenyl) 0,0-Dimethyl Ester	Rat	330	790	2.39		
Phosphorothioic Acid, 0 (3-Chloro-4-Nitrophenyl) 0,0-Dimethyl Ester	Rat	880	1,500	1.71		

APPENDIX (Continued)
Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD_{50} (mg/kg)	Dermal LD_{50} mg/kg	Dermal/Oral	Rabbit/Rat	
Phosphorothioic Acid, S-(((Cyano-1-Methyl-Ethyl)Carbamoyl)Methyl) O,O-Diethyl Ester	Rat	3.5	105	30.0		
Phosphorothioic Acid, O-(2,5-Dichloro-4-Iodophenyl) O,O-Dimethyl Ester	Rat	2,000	1,800	0.90	Oral	1.00
	Rabbit	2,000	500	0.25	Dermal	0.28
Phosphorothioic Acid, O,O-Diethyl O-((2,5-Dichloro-4-Methylthio)Phenyl) Ester	Rat	7.8	58	7.44	Oral	2.56
	Rabbit	20	48	2.40	Dermal	0.83
Phosphorothioic Acid, O,O-Diethyl O-(2-(Diethylamino)-6-Methyl-4-Pyrimidinyl) Ester	Rat	140	1,000	7.14		
Phosphorothioic Acid, O,O-Diethyl Ester, O.O-Diethyl ESter, O-Ester with 6-Hydroxy-2-Phenyl-3(2H)Pyridazinone	Rat	850	2,100	2.47		
Phosphorothioic Acid, O,O-Diethyl O-(2-(Ethylthio)Ethyl) Ester, mixed with O,O-Diethyl S (2-(Ethylthio)Ethyl) Ester (7:3)	Rat	1.7	8.2	4.82	Dermal	2.93
	Bird (Wild)	7	1.8	0.26		
	Rabbit	--	24	--		
Phosphorothioic Acid, O,O-Diethyl O-(2-Isopropyl-6-Methyl-4-Pyrimidinyl) Ester	Rat	76	455	5.99	Dermal	0.88
	Rabbit	-	400	--		
Phosphorothioic Acid, O,O-Diethyl O-(p-(Methylsulfinyl)Phenyl) Ester	Duck	7.47	3	0.40		
	Bird (Wild)	2.4	4.2	1.75		
	Rat	2	3	1.50		
Phosphorothioic Acid, O,O-Diethyl O-(p-Nitrophenyl) Ester	Rat	2	6.8	3.40	Oral	5.00
	Mouse	6	32.4	5.40	Dermal	5.88
	Rabbit	10	40	4.00		
	Guinea Pig	8	600	75.0		
	Duck	2.34	28	12.0		
	Bird (Wild)	2	1.80	0.90		

APPENDIX (Continued)
Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD$_{50}$ (mg/kg)	Dermal LD$_{50}$ mg/kg	Dermal/Oral	Rabbit/Rat
Phosphorothioic Acid, 0,0-Diethyl 0-(5-Phenyl-3-Isoxazolyl) Ester	Rat	112	450	4.02	
	Mouse	98	193	1.97	
Phosphorothioic Acid, 0,0-Diethyl 0-(1-Phenyl-1,2,4-Triazolyl) Ester	Rat	64	1,100	17.2	
Phosphorothioic Acid, 0,0-Diethyl 0-Pyrazinyl Ester	Rat	3.50	8	2.29	
	Duck	1.68	74.17		
Phosphorothioic Acid, 0,0-Diethyl 0-(2-Quinoxalinyl) Ester	Rat	26	300	11.5	
Phosphorothioic Acid, 0,0-Diethyl 0-(3,5,6-Trichloro-2-Pyridyl) Ester	Rat	97	202	2.08	Oral 10.31
	Rabbit	1,000	2,000		Dermal 9.90
Phosphorothioic Acid, 0,0-Dimethyl Ester, 0,0-Diester with 4,4'-Thiodiphenol	Rat	1,000	1,370	1.37	Dermal 0.73
	Rabbit	--	1,000	-	
Phosphorothioic Acid, 0,0-Dimethyl Ester, 0-Ester, 0-Ester with p-Hydroxybenzonitrile	Rat	18	800	44.4	
Phosphorothioic Acid, 0,0-Dimethyl Ester S-Ester with 2-((2-Mercaptoethyl)Thio)-N-Methylpropionamide	Mouse	40	1,500	37.5	
	Rabbit	-	160	--	
Phosphorothioic Acid, 0,0-Dimethyl Ester, S-Ester with 2-(Mercaptomethyl) -5-Methoxy-4H-Pyran-4-One	Rat	23	130	5.65	
Phosphorothioic Acid, 0,0-Dimethyl S-(2-(Ethylsulfonyl)Ethyl) Ester	Rat	40	500	12.5	
Phosphorothioic Acid, 0,0-Dimethyl S-(2-(Methylthio)Ethyl) Ester	Rat	84	68	0.81	
Phosphorothioic Acid, 0,0-Dimethyl-, 0-(4-Methylthio)-m-Tolyl) Ester	Rat	215	330	1.54	
	Duck	6	44	7.33	

APPENDIX (Continued)
Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD_{50} (mg/kg)	Dermal LD_{50} mg/kg	Dermal/Oral	Rabbit/Rat
Phosphorothioic Acid, O,O-Dimethyl O-(p-Nitrophenyl) Ester	Rat	6	63	10.5	Oral 70.00
	Rabbit	420	300	0.71	Dermal 4.76
	Duck	61	54	0.88	
Phosphorothioic Acid, O,O-Dimethyl O-(4-Nitro-m-Tolyl) Ester	Mouse	715	2,500	3.50	
	Duck	1,190	504	0.42	
Phosphorothioic Acid, O,O-Dimethyl O(2,4,5-Trichlorophenyl) Ester	Rat	906	2,000	2.21	Oral 0.46
	Rabbit	420	1,000	2.38	Dermal 0.50
	Guinea Pig	1,4000	2,000	1.43	
Phosphorothioic Acid, O-Ethyl S-Propyl O-(2,4,6-Trichlorophenyl) Ester	Rat	200	250	1.25	
Phosphorothioic Acid, S-(2-(Ethylsulfinyl)Ethyl) O,O-Dimethyl Ester	Rat	30	100	3.33	
Phosphorothioic Acid, S-(2-(Ethylsulfinyl)-1-Methylethyl) O,O-Dimethyl Ester	Rat	103	1,000	9.71	
Phosphorothioic Acid, O-(2-(Ethylthio)Ethyl) O,O-Dimethyl Ester, mixed with Phosphorothioic Acid, S-(2-(Ethylthio)Ethyl) O,O-Dimethyl Ester (7:3)	Rat	65	300	4.62	
Phosphorotrithioic Acid, S,S,S-Tributyl Ester	Rat	150	168	1.12	
Phosphorotrithious Acid, Tributyl Ester	Rat	910	615	0.68	
Phosphorotrithious Acid, Trimethyl Ester	Rat	105	1,030	9.81	
Phthalic Acid, Bis(2-Ethylhexyl) Ester	Rabbit	3400	2,500	0.74	
Phthalimide, N-(2,6-Dioxo-3-Piperidyl)-	Rat	113	1,550	13.7	

APPENDIX (Continued)
Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD_{50} (mg/kg)	Dermal LD_{50} mg/kg	Dermal/Oral	Rabbit/Rat
Piperidinium, 1-Allyl-1-(3,7-Dimethyloctyl)-, Bromide	Rat	360	115	0.32	
Pivalic acid	Rat	900	1.900	2.11	
Poly(Oxy(Methyl-1,2-Ethanediyl)), alpha-Butyl-omega-Hydroxy-	Rabbit	23,900	2,100	0.09	
Polypropylene Glycol Monobutylether	Rabbit	23,900	2,100	0.09	
Propane, 1-Chloro-2-Nitro-	Rat	197	362	1.84	Dermal 1.00
	Rabbit	--	362	--	
Propane, 1,2-Dibromo-3-Chloro-	Rabbit	180	1,400	7.78	
Propane, Dichloro- mixed with Propene, Dichloro-	Rat	140	2,100	15.0	Dermal 1.00
	Rabbit	--	2,100	-	
1,3-Propanediol, 2-Ethyl 2-(Hydroxymethyl)-, Cyclic Phosphate	Rat	3.08	50	16.2	
1,3-Propanediol, 2-Ethyl-2-(Hydroxymethyl)-, Cyclic Phosphite (1:1)	Rat	8.39	929	110	
	Mouse	7	4	0.57	
2-Propanol, 1-methoxy-	Rabbit	8,000	13,000	1.63	
2-Propanone, Chloropentafluoro-, Hydrate	Rat	85	81	0.95	
2-Propanone, 1,3-Dichloro-1,1,3,3-Tetrafluoro-	Rat	61	91	1.49	
2-Propanone, 1,1,1,3,3,3 Hexachloro-	Rat	1,290	2,980	2.31	Dermal 1.00
	Rabbit	--	2,980	--	
2-Propanone, Hexafluoro-, Hydrate	Rat	190	113	0.60	
2-Propanone, 1,1,3-Trichloro-1,3,3-Trifluoro-	Rat	277	770	2.78	Dermal 1.00
	Rabbit	--	770	-	

APPENDIX (Continued)
Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD$_{50}$ (mg/kg)	Dermal LD$_{50}$ mg/kg	Dermal/Oral	Rabbit/Rat	
Propionaldehyde, 2-Methyl-2-(Methylsulfonyl)-, O-Methylcarbamoyl)-Oxime	Rat	26.8	1,000	37.3	Dermal	1.00
	Rabbit	--	1,000	-		
Propionaldehyde, 2-Methyl-2-(Methylthio)-, O-(Methylcarbamoyl)-Oxime	Rat	900	2.5	0.003		
Propionic Acid, 2-Chloro-3-(4-Chlorophenyl)-, Methyl Ester	Rabbit	500	756	1.51		
Propionic Acid, 2-(2,4-Dichlorophenoxy)-	Mouse	400	1,400	3.50		
	Rat	800	1,400	1.75		
Propionitrile, 2-((4-Chloro-6-Ethylamino)-s-Triazin-2-Ylamino)-2-Methyl-	Rat	149	1,200	0.05		
Pyridine, 2-Chloro-6-(Trichloromethyl)-	Rabbit	500	850	1.70		
2,5-Pyridinedicarboxylic Acid, Dipropyl Ester	Rat	5,230	9,400	1.80	Dermal	1.01
	Rabbit	-	9.500	-		
Pyrophosphoric Acid, Tetraethyl Ester	Rat	0.5	2.4	4.80	Dermal	2.08
	Duck	3.56	64	18.0		
	Rabbit	--	5	--		
Pyrophosphoric Acid, Tetraethyl Ester (liquid mixture)	Rat	1.05	2.40	2.29		
Pyrrolidine, 1-Butyl-	Mouse	51	1,000	19.6		
Ryania	Rat	750	750	1.00		
Serine, p-nitrophenyl-	Rat	24,000	16,000	0.67		
Succinic Acid, Mercapto-, Diethyl Ester, S-ester with O,O-Dimethyl Phosphorodithioate	Rabbit	250	4,100	16.4	Oral	0.28
	Rat	885	4,444	5.02	Dermal	0.92

APPENDIX (Continued)
Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD_{50} (mg/kg)	Dermal LD_{50} mg/kg	Dermal/Oral	Rabbit/Rat	
Sulfamide, N-((Dichlorofluoromethyl)-Thio)-N',N'-Dimethyl-N-(p-Tolyl)-	Rat	1,000	500	0.50		
1,2,4-Thiadiazole, 5-Ethoxy-3-(Trichloromethyl)-	Rabbit	779	1,700	2.18		
Thiocyanic Acid, 2-(2-Butoxyethoxy) Ethyl Ester	Rat	90	250	2.78	Oral	0.39
	Rabbit	35	125	3.57	Dermal	0.50
Thiopyrophosphoric acid, Tetrapropyl ester	Rat	450	1800	4.00	Dermal	2.13
	Rabbit	--	3830	--		
as-Triazin-5(4H)-ONE, 4-Amino-6-tert-Butyl-3-(Methylthio)-	Rat	2,200	2,000	0.91		
Tributylamine	Rabbit	615	250	0.41		
Triethylenetetramine	Rabbit	5,500	820	0.15		
s-Trioxane, 2,4,6-trimethyl-	Rabbit	3,304	14,000	4.24		
1,3,5,2,4,6-Triphosphatriborin, 1,2,3,4,5,6-Hexahydro-1,2,3,4,5,6-Hexamethyl-	Rat	13.5	1,800	133		
m-Toluamide, N,N-diethyl-	Rat	1,950	5,000	2.56	Oral	0.81
	Rabbit	1,584	3,180	2.01	Dermal	0.64
Toluene, alpha-(2-(2-Butoxyethoxy) Ethoxy)-4,5-(Methylenedioxy)-2-Propyl-	Rabbit	7,500	200	0.03	Dermal	1.71
Toxaphene	Rat	40	600	15.0		
	Rabbit	--	1,025	--		
x Triazine, 2-Chloro-4-Ethylamino-6-Isopropylamino-	Rabbit	750	7,500	10.0		
v-Trithiane, 5-(Dimethylamino)-, Oxalate	Rat	310	1,000	3.23		
Urea, 3-(Hexahydro-4,7-Methanoindan-5-YL)-1,1-Dimethyl-	Rat	2,000	23,000	11.5		

APPENDIX (Continued)

Comparison of Oral and Dermal Lethality

Chemical	Species	Oral LD_{50} (mg/kg)	Dermal LD_{50} mg/kg	Dermal/Oral	Rabbit/Rat
2,4-Xylenol	Rat	3,200	1,040	0.33	
2,6-Xylenol	Rabbit	700	1,000	1.43	
	Mouse	980	920	0.94	